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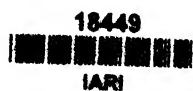
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# ON THE STRUCTURE OF ANAEROBIC BACTERIA

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Our notions concerning the structure of bacteria lag considerably behind our knowledge in other fields of cytology. In the course of the last thirty to forty years the study of the cell has resulted in a series of most important discoveries and has led to the creation of such new, independent branches of cytology as cytogenetics and cytophysiology. But investigations leading to the exposition of the nature of different cellular inclusions have not yet been completed for the cytology of microorganisms. The small dimensions of the cells, the rapid changes of their structures in the process of ontogenesis connected with an accelerated growth and propagation, as well as a series of other reasons, make it difficult to study the structure of bacteria.

It is probable that there exists a dependence between the physiological peculiarities of microorganisms and the structure of their cells, that is, the principal functions of the microbe must be reflected in detailed cytological peculiarities. A different structure of the cells must correspond to a distinct physiological specialization.

So it is from the point of view of theory that, taking into account the present condition of cytology, these peculiarities of the bacterial cell structure remain unknown.

In our investigations of the structure of microbes we used the comparative morphological method which permitted us to determine a gradual complication of the nuclear apparatus of various representatives of the *Myxobacterales* and to note some peculiarities in the structure of the *Eubacterales* (Imšenecki, 1940). Continuing our investigations we compared the structure of aerobic and anaerobic bacteria which form spores.

Among sporogenous bacteria, chiefly aerobic varieties have previously been studied (Guilliermond, 1908; Hollande, 1934; and others). And if the structure of cells of spore-building anaerobic forms was studied, no peculiarities whatever were noted by many workers. In works dealing with the cytology of microbes the structure of bacilli in general is described without differentiating between aerobic and anaerobic species. Meanwhile, on the basis of theoretical considerations, one could suppose that the character of the metabolism had to lay its imprint on the structure of the anaerobic cells.

We may point out a series of morphological symptoms according to which it is sometimes possible to distinguish aerobic bacteria from anaerobic ones. All these symptoms, however, are casual and therefore have but a relative value. Here among the biological peculiarity of an undoubtedly adaptive character of forming filaments and chains of cells composing the films which are formed in cultures of aerobic sporogenous bacteria on liquid media. This symptom loses



its significance for the aerobic species which do not form chains of cells and films.

Further let us note that the aerobic bacteria have a greater cell diameter than the anaerobic species, the vegetative cells of which are usually thinner and more refined. A change in the outlines of the cells during spore formation specifically, the production of clostridial and plectridial forms, is more often observed in the anaerobic species. All these symptoms, being variable, cannot be used for the differentiation of aerobes from anaerobes. The specificity of interest to us should be searched for in the finer peculiarities of the structure of the cells themselves.

The data reported in this paper are of a preliminary character; a more detailed analysis of the described facts will be given later.

#### CYTOLOGICAL INVESTIGATIONS

Aerobic and anaerobic sporogenous bacteria were submitted to a comparative study. We took the following aerobic species: *Bacillus mycoides*, *Bacillus tumescens*, *Bacillus megatherium*, *Bacillus mesentericus*, and *Bacillus ellenbachensis*. As representatives of the anaerobic species we examined obligate anaerobes which usually do not develop on the surface of solid media under ordinary conditions. They were *Clostridium sporogenes*, *Clostridium perfringens*, *Clostridium amylobacter*, *Clostridium acetobutylicum*, and *Clostridium putrificum*, as well as mesophilic and thermophilic varieties of sporogenous bacteria which decomposed cellulose. Moreover, we examined two strictly anaerobic species of bacilli freshly isolated from horse dung; thereby the cytological analysis of these laboratory cultures was completed by the study of bacteria isolated immediately from nature.

The cultivation of aerobic and anaerobic bacteria was effected on media of the same composition. The media used were flesh peptone agar with glucose, liver agar with glucose, and potato agar. For cellulose-fermenting bacteria we used special media the composition of which was given in a previous paper (Imšenecki, 1939).

Aerobic bacilli were cultivated on the surface of media, anaerobic ones in agar "columns" or on the surface of solid media in metal exsiccators from which the air had been pumped out. The cells were examined microscopically from cultures 2, 3, 4, and 6 days old.

The most characteristic peculiarity of the structure of aerobic bacteria is the presence of bodies consisting of lipoproteins. They represent bright spheric inclusions well distinguished in unstained cells. The lipoprotein corpuscles, being small in young cells, become larger in older cultures and, when increasing, often unite. Then the corpuscles form the shape of the figure eight apparently preceding the division or fission of the corpuscles. Owing to the presence of inclusions, the content of the cells of aerobic bacilli has a granular aspect; 2 to 4 bright corpuscles of different dimensions may be seen in the cell. On solid media containing glucose or starch, inclusions may reach large sizes and the cell seems to be filled with them.

The lipoprotein inclusions in the unfixed cells are dissolved by acetic acid or acetone and not dissolved in alcohol, ether, or chloroform. The corpuscles are stained well with *para*-phenylenediamine and with Sudan III. By the staining of fixed preparations with the usual methods, the large lipoprotein inclusions are not stained, and empty places resembling vacuoles may be observed in the cells. However, a previous post vital treatment of the cells with picric acid or  $\alpha$ -naphthol easily permits staining the inclusions with basic dyes (fuchsin, gentian violet, or dahlia).

The lipoprotein corpuscles consist chiefly of fats and lipoids, and represent structures in which reserve substances are stored. They disappear during starvation of the cells, but considerably increase in size when the bacteria are grown on media with carbohydrate. The formation of these inclusions is a physiological phenomenon, and it differs completely from the appearance of granularity observed during the pathological splitting of the lipoprotein complex of the protoplasm (lipophaneroze) which takes place because of different reasons.

The corpuscles described may be observed in the cells of all sporogenous bacteria; it is the most constant intracellular structure. When studying the cytology of bacteria, investigators, unfortunately, are searching only nuclei and pay little attention to the inclusions in the bacterial cell, the dimensions of which are determined entirely by the state of nutrition. Because of the fact that lipoprotein corpuscles stain with basic dyes by some methods, and by fusing present pictures resembling corpuscle division by amitosis, such bodies have often been considered nuclei. A number of investigators have described these inclusions as nuclei. (For comprehensive reviews of this subject, see Meyer, 1912; Knaysi, 1938; Imšenecki, 1940; and Lewis, 1941.)

We shall give here but a short account of the inclusions so far as these investigations have confirmed the peculiarities of the corpuscles described previously (Imšenecki, 1940). We shall emphasize that the lipoprotein corpuscles are structures most typical for aerobic sporogenous bacteria. They are to be found in the cells of all aerobic sporogenous bacteria investigated by us and are formed in the cells independently of the contents of the medium; on substrates rich in carbohydrates they increase in size.

Let us now deal with the cytology of anaerobic bacilli. Chromatin in the cells of both aerobic and anaerobic bacilli is distributed diffusibly in the protoplasm. Before spore formation the chromatin precipitates out from the cytoplasm and forms a small chromophilic corpuscle (prospore) which is gradually transformed into a mature spore. The morphological changes corresponding to different stages of this process are by no means specific; the cytology of spore formation is similar for aerobes and anaerobes.

The majority of anaerobic sporogenous bacteria have slender cells; the lengths of the rods as a rule do not differ from those of the cells of aerobic species, but they are thinner. This characteristic, however, though marked, does not serve as a reliable criterion.

During the microscopic examination of living cells of anaerobic bacteria,

both from young and old cultures, one notes the exceptional homogeneity of the contents of the bacteria. The cytoplasm of vegetative cells is homogeneous, opaque, and entirely devoid of lipoprotein inclusions. The results of observations *in vivo* are confirmed by the microscopic examination of preparations stained by various methods.

Neither observations of living cells, microchemical reactions, nor special staining permit observation of lipoprotein corpuscles in anaerobes. Investigations were pursued with cultures of anaerobic bacilli grown on media rich in carbohydrates, but even in that case we could not confirm the formation of lipoprotein inclusions in the cells of either of the species examined.

It should be noted that in very old cultures of anaerobes, cells are to be found with their contents fragmented into separate sections. In the cell we find from 2 to 5 granules which can be intensely stained. These morphological pictures, noted by us as pseudoplasmolysis, correspond to the dying off and decay of the cells and have nothing in common with the physiological storage of reserve substances.

The absence of bright corpuscles in the cells of anaerobic bacteria and, on the other hand, their constant presence in the aerobic ones may explain why they have a different structure in certain phases of their individual development. Thus, the prospore forming in the cell of aerobic bacteria seems to drive the lipoprotein corpuscles back to the opposite side of the cell. During that period the bright inclusions accumulate in the part of the cell free from the prospore. The anaerobes, on the contrary, form the spore in a cell completely devoid of bright lipid or fat inclusions during all the stages of that process. In that way, aerobic and anaerobic bacteria may be differentiated on the basis of their cytological peculiarities.

Reserve substances in the form of corpuscles consisting of fat and lipoids quickly accumulate in the cells of the aerobes. The propagation of cells as well as the formation of lipoprotein corpuscles does not take place under anaerobic conditions. The necessity of aeration for the intensive formation of fat is characteristic not only for bacteria but also for yeast, such as some *Endomyces* and *Torulopsis*. The generation of corpuscles, representing a physiological phenomenon, is connected with the storage of sources of energy; this is confirmed by the fact that bacteria consume fat inclusions during starvation. However, the lipoprotein corpuscles are used chiefly by means of oxidizing the fat; i.e., a process which, like the formation of these inclusions, develops most intensively under aerobic conditions. On the contrary, the oxidation processes connected with the consumption of fat inclusions, stored in the cells of the aerobes, cannot take place in the cells of anaerobic species of bacteria. The anoxibiontes have difficulty in consuming fat in the cell protoplasm. If we consider anaerobiosis as an adaptation effected by aerobes, then the anaerobic bacteria were generated from aerobic bacteria. Anaerobic conditions favor anaerobic forms with the metabolic peculiarities found in the absence of lipoprotein inclusions.

As is known, some sporogenous bacteria store carbohydrates (granulose,

starch, iogen) as reserve substances; their presence in the cell usually precedes the stage of spore formation. These kinds of inclusions chiefly occur in the cells of anaerobic bacteria. The quantity of these reserve substances decreases during the process of spore formation, as may be distinctly seen in the case of the so-called *Granulobacter pectinovorum*. Evidently the carbohydrates of anaerobic bacteria may easily serve as a source of energy, the more so because, as a rule, the storage of these reserve substances takes place in bacteria which are able to ferment analogous carbohydrates when present in their growth medium.

The facts concerning the differences in structure of aerobic and anaerobic bacilli revealed by these investigations confirm the general statement that function in microbes is reflected in the structure of the cells. It is possible that further investigations will broaden our conceptions of this subject, and that other peculiarities of the structure of aerobic and anaerobic bacteria will be found, but the cytological characteristics described above permit the differentiation of these two groups of organisms.

#### SUMMARY

The comparative cytological study of aerobic and anaerobic bacteria which form spores has shown that their cytology is different. In the cells of aerobic bacilli, lipoprotein corpuscles, representing inclusions of reserve substances, are constantly present. Anaerobic bacteria do not contain such corpuscles. The generation of lipoprotein corpuscles, as well as the consumption of the fat contained in them, is closely connected with aerobic conditions of life.

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# MICROBIOLOGICAL ASPECTS OF PENICILLIN

## VII. BACTERIAL PENICILLINASE

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It is common experience to have penicillin culture broths prove susceptible to bacterial contamination, this usually being accompanied by inactivation of the penicillin. Abraham and Chain (1940) have shown that certain bacteria produce an enzyme named penicillinase, which destroys penicillin. The penicillin resistance of some bacteria was ascribed to their ability to produce penicillinase, but this explanation alone did not apply to other bacteria. Harper (1943) made acetone dried bacterial preparations suitable for the inactivation of penicillin to permit detection of sensitive organisms which otherwise would be inhibited during routine testing of infected body fluids containing penicillin. The penicillin-destroying powers of commercial clarase and takadiastase are due to their content of aerobic, sporeforming, penicillinase-producing bacteria (Lawrence, 1944).

The penicillin used in this work contained usually 100 to 200 Oxford units per milligram.

### *Destruction of penicillin by fully developed microbial cultures*

**Bacteria.** Several pure cultures of bacteria (table 1) were grown for 24 hours at 37 C in nutrient broth. Two species of actinomycetes, grown with shaking in yeast extract, were also included (Woodruff and Foster, 1943). The fully developed cultures were adjusted to pH 7.0, penicillin was added to a final concentration of 800 Oxford units per ml, and the mixture was allowed to stand at 37 C for 2 hours. Residual penicillin was determined either turbidimetrically or by the *Bacillus subtilis* cup method (Foster, 1942; Foster and Woodruff, 1943b). Table 1 shows that the ability to destroy penicillin is widespread among different bacteria, this property being particularly marked in the case of the aerobic sporeformers.<sup>1</sup> Thus, *Bacillus cereus*, *Bacillus megatherium*, and *Bacillus mesentericus* were clearly the strongest penicillin decomposers, destroying all the available penicillin in 2 hours. The destruction of all the penicillin by the two species of *Actinomyces* is worthy of note. *Staphylococcus aureus* H, the strain first used in penicillin work at Oxford (Abraham *et al.*, 1941; Foster and Woodruff, 1943a), is extremely sensitive to penicillin action, yet it decomposed as much penicillin as *Escherichia coli*. Kirby (1944) has since shown that extracts of resistant strains of *S. aureus* contain penicillinase. Also, although *Pasteurella* sp. is one of the most sensitive gram-negative rod-

<sup>1</sup> In some cases the destruction may not be significant due to assay inaccuracies which can have as much as 25 per cent variation (compare, e.g., *S. aureus* FDA with control in table 1), but in most cases the destruction was beyond question.

shaped bacteria to penicillin inhibition, it appeared to have considerable capacity for penicillin destruction. The same is true of the very sensitive *Micrococcus lysodeikticus*. Such results re-emphasize the findings of Abraham and Chain (1940) about the lack of relation between resistance and enzyme formation by the organisms. However, *Mycobacterium tuberculosis*, which is virtually insensitive to penicillin inhibition, is perhaps so because it is one of the most active destroyers of penicillin.

*Yeasts.* These were treated the same as the bacteria except that 72-hour-old yeast extract broth containing 1 per cent glucose was used as the medium. In each of the following cultures, almost all of the 800 units of penicillin per ml initially present remained after the incubation at 37 C: *Candida stellatoidea*, *Saccharomyces cerevisiae*, *Schwanniomyces occidentalis*, *Torulaspora delbrueckii*,

TABLE 1  
Destruction of penicillin by various bacteria

CULTURE	PENICILLIN REMAIN- ING
	Oxford units per ml
Uninoculated control.....	800
<i>Pseudomonas aeruginosa</i> .....	670
<i>E. coli</i> .....	550
<i>Pasteurella</i> sp.....	250
<i>S. aureus</i> H.....	550
<i>S. aureus</i> FDA.....	920
<i>Micrococcus lysodeikticus</i> .....	430
<i>B. subtilis</i> .....	130
<i>B. cereus</i> .....	0
<i>B. megatherium</i> .....	0
<i>B. mesentericus</i> .....	11
<i>Bacillus</i> W.....	340
<i>Mycobacterium tuberculosis</i> *.....	0
<i>Actinomyces lavendulae</i> .....	0
<i>Actinomyces antibioticus</i> .....	0

\* Seven-day-old culture.

*Rhodotorula rubra*, *Sporobolomyces roseus*, *Zygopichia californica*, *Torulopsis menosa*, and *Zygosaccharomyces marxianus*.

Only two yeasts were fairly effective in destroying penicillin, *Mycoderma valida* and *Debaryomyces guilliermondii* inactivating 68 and 81 per cent, respectively. The approximately 20 per cent reduction observed in the case of most of the other organisms probably was due to loss accompanying removal of the cells during centrifugation. Probably diffusion into the cells and physical adsorption were responsible.

*Fungi.* The fungus tests were similar to those with yeasts except that the fungi were grown with shaking for three days in yeast-extract glucose broth, and the penicillin was added to the neutralized whole cultures. The following fungi (the majority of them from Dr. Charles Thom) were tested: *Aspergillus*

*flavus*, *Aspergillus clavatus*, *Penicillium notatum* 77, *P. notatum* 832, *Penicillium chrysogenum*, *Gliocladium deliquescens*, *Monascus ruber*, *Monilia sitophila*, *Fusarium moniliformis*, *Acrothecium robustum*, *Spondylocadium xylogenum*, *Mucor* sp., *Helminthosporium* sp., *Neurospora* sp., *Chaetomium* sp., *Paecilomyces* sp., *Papulaspora* sp., *Sepedonium* sp., *Dematium* sp., *Syncephalastrum* sp., *Basisporium* sp., *Alternaria* sp., *Trichoderma* sp., *Metarrhizium* sp., and *Cladosporium* sp. As a group, the fungi were relatively weak in their penicillin-destroying powers under the conditions of the experiment. The slight reductions observed in all except one culture may be ascribed to diffusion into and physical absorption by the cells. The single exception was *Papulaspora* sp. which, in three separate tests, destroyed 800 units of penicillin within 2 hours.

TABLE 2

*Extracellular penicillinase formation by various bacteria in different media*

CULTURE	MEDIUM	PENICILLIN LEFT	PENICILLIN DESTROYED
		units per ml	per cent
	None (control)	36	—
W1	Yeast extract	11	69
W1	Yeast extract + glucose	25	31
W1	Nutrient broth	5	81
W1	<i>P. notatum</i> medium	15	58
W3	Yeast extract	5	81
W3	Yeast extract + glucose	31	14
W3	Nutrient broth	15	58
W3	<i>P. notatum</i> medium	34	6
164	Yeast extract	0	100
164	Yeast extract + glucose	28	22
164	Nutrient broth	0	100
164	<i>P. notatum</i> medium	0	100
184	Yeast extract	18	50
184	Yeast extract + glucose	34	6
184	Nutrient broth	34	6
184	<i>P. notatum</i> medium	34	6

Enzyme solution concentration = 20 per cent by volume; time = 1 hr; temp = 37C.

*Production of extracellular penicillinase by Bacillus sp.*

*Bacillus* sp. was isolated from a contaminated culture of *Penicillium notatum*. The cells in four-day-old nutrient broth cultures of *Bacillus* sp. incubated at 37 C were removed by centrifugation at 4,500 rpm. Seitz filtration was not used to remove the cells because it was shown that part or all of the enzyme activity may be lost by this, presumably by adsorption. Microscopic examination showed that the supernatant was cell-free. This liquid actively destroyed penicillin. The enzyme solution containing 14 units of added penicillin per ml was incubated at 37 C (pH 7.0) for various time intervals after which the solutions were pasteurized to halt enzyme action. Penicillin itself is little affected



by such heating (Foster and Wilker, 1943). Twenty minutes of incubation were sufficient for the destruction of all the penicillin present.

*Extracellular penicillinase formation by various bacteria in different media*

Table 2 demonstrates the comparative ability of four newly isolated but unidentified species of bacteria to produce extracellular penicillinase when grown in four different media. These organisms were isolated from contaminated penicillin-containing solutions. Cultures W1, W3, and 184 were aerobic spore-forming bacilli isolated by selective culture technique on penicillin-containing plates. Culture 164 was an aerobic sporeforming bacillus tentatively identified through diagnostic media as belonging to the *B. subtilis* group. *Bacillus* 164 clearly was most active when tested in 20 per cent concentration for 1 hour at 37 C. In each case the presence of glucose in yeast extract broth greatly reduced the penicillin-destroying ability of the cell-free medium as compared to plain yeast extract.

*Effect of sugars and penicillin on extracellular penicillinase formation*

This effect was studied more closely with culture no. 164. One-half of each of two series of media, yeast extract and yeast extract containing 1 per cent glucose, received daily a sterile penicillin solution equivalent to 5 units per ml final concentration. The other half received none. All were inoculated with culture 164, incubated at 37 C and duplicate samples removed daily for determination of extracellular penicillinase activity. Each solution was tested against penicillin for 1 hour at 37 C in concentrations of 0.2, 2, and 20 per cent by volume. Table 3 confirms the reduction in penicillinase content of glucose-containing media. Also, penicillinase was produced by the bacteria just as much in the absence of penicillin as in its presence, and it may, therefore, be considered as a constitutive enzyme (Karström, 1937). Enzyme activity of sugar-free supernatant culture fluid was at a maximum within 24 hours. It declined slowly with continued incubation, but even after 120 hours an appreciable amount of enzyme remained. In the glucose media, maximum activity appeared at the third day. Of other sugars tested, including mannose, galactose, lactose, sucrose, xylose, arabinose, and mannitol, only glucose and fructose reduced penicillinase formation, and, interestingly, the fall in the pH of the medium was greatest in these two and in direct relation to the reduction in penicillinase activity. Thus, the glucose supernatant destroyed 22 per cent of the available penicillin, and its pH was 5.2; fructose supernatant destroyed 62 per cent, and its pH was 5.8. All the others destroyed all the available penicillin, and their pH values were 6.1 or higher. These results could not be due to a retardation of the action of the enzyme itself, since in each case the enzyme solution was adjusted to pH 7.0 before testing.

*pH and extracellular penicillinase formation*

This apparent relation between pH and the content of extracellular penicillinase led to a more detailed study. Table 4 shows the effect of initial pH on

formation of the enzyme in the presence of two different glucose concentrations in 16-hour cultures. Sörenson phosphate buffer at various pH levels was added to a final concentration of  $M/15$ . The results point to pH being the controlling

TABLE 3

*Extracellular penicillinase production in glucose- and penicillin-containing cultures of Bacillus 164*

MEDIUM	DAILY PENICILLIN ADDITION TO MEDIUM	CONCENTRATION OF CELL-FREE ENZYME SOLUTION	PENICILLIN DESTROYED*			
			24 hr	48 hr	72 hr	120 hr
		volume, per cent	per cent	per cent	per cent	per cent
Yeast extract	—	20	>75	>75	>75	>75
	—	2	72	71	39	24
	—	0.2	21	0	0	2
Yeast extract	5 units/ml	20	—	>75	>75	>75
	5 units/ml	2	—	>75	0	55
	5 units/ml	0.2	—	0	0	0
Yeast extract + glucose	—	20	32	32	59	7
	—	2	21	0	12	0
	—	0.2	6	0	0	0
Yeast extract + glucose	5 units/ml	20	—	32	>40	7
	5 units/ml	2	—	0	0	0
	5 units/ml	0.2	—	0	0	0

\* Initial concentration = 16 units per ml.

TABLE 4

*pH and penicillinase formation*

INITIAL pH (BUFFERED)	NO GLUCOSE		0.1% GLUCOSE			0.5% GLUCOSE		
	Final pH	Penicillin* destroyed	Final pH	Glucose left	Penicillin destroyed	Final pH	Glucose left	Penicillin destroyed
		per cent		mg/ml	per cent		mg/ml	per cent
7.6	7.7	100	7.4	0.31	100	6.5	0.32	85
7.1	7.5	100	6.8	0.20	100	—	—	—
6.2	6.4	100	6.1	0.31	100	5.5	0.47	10
5.6	6.1	78	5.8	0.29	100	5.3	1.79	8
4.5	5.8	55	5.4	0.29	10	5.5	1.89	10
No buffer	6.4	100	6.1	0.28	50	5.6	3.76	0

\* Initial concentration of penicillin = 20 units per ml.

factor in extracellular penicillinase formation by this organism. In no case was there more than a trace of activity when the reaction was pH 5.5 or less although abundant bacterial growth did proceed at acidity values down to at least pH 5.3 as evidenced by visible turbidity and sugar utilization. In the

unbuffered control, in the presence of glucose, the pH fell to a point where it limited penicillinase formation, whereas in the buffered sets (5.6 or higher) acid formation from the glucose had no effect on enzyme production, due to neutralization by the buffer.

*Bacillus* 164 is uninhibited in growth by at least several hundred units of penicillin per ml of culture liquid at pH 6.0 or above, because under these conditions it destroys the penicillin by virtue of penicillinase. However, when grown at pH 5.5, where it cannot produce penicillinase, the organism is definitely subject to penicillin inhibition, growth being completely suppressed by 10 units per ml.

The absence of formation of penicillinase at pH 5.5 or less led to some attempts to suppress destruction of penicillin in contaminated *Penicillium notatum* cultures. These experiments were unsuccessful, since penicillin formation by the mold does not occur in such acid media (Abraham *et al.*, 1941; Foster *et al.*, 1943).

TABLE 5  
*Penicillinase concentration-time relationship*

CONCENTRATION	PENICILLIN* DESTROYED, PER CENT				
	5 min	30 min	60 min	120 min	240 min
<i>volume per cent</i>					
99	50	100	100	100	100
50	19	100	100	100	100
25	0	100	100	100	100
12.5	0	95	100	100	100
6.3	0	69	95	100	100
3.1	0	47	55	100	100
0	0	0	0	0	0

\* Initial content of penicillin = 33 units per ml.

*Properties of extracellular penicillinase from culture 164*

The concentration-time relationship of this enzyme solution (yeast extract medium) is given in table 5. A proportionality exists between the amount of enzyme and the rate of penicillin inactivation. Thus 3 per cent by volume of enzyme solution inactivated all 33 units of penicillin per ml in 120 minutes. Twice this amount of enzyme required only 60 minutes and 4 times this amount required just  $\frac{1}{2}$  the incubation period.

*Temperature stability of penicillinase*

Aliquots of the cell-free enzyme solution (adjusted to pH 7.0) were allowed to stand 30 minutes in water baths at temperatures differing by 5 C from 35 to 65 C. One treatment was run at 4 C. Enzyme activity was then measured as usual at 37 C with 44 units of penicillin per ml present initially. The enzyme was completely destroyed at 50 C and above, whereas there was no loss from 45 C down to 4 C—at which temperature the enzyme is stable for months.

*pH stability of penicillinase*

Aliquots of the cell-free enzyme solution were adjusted to different pH values with HCl or NaOH, allowed to stand 30 minutes at 37 C, neutralized and tested for enzyme activity. Over a pH range of 3.0 to 11.0 the enzyme maintained its full activity. This wide range appears to be unusual for an enzyme. It was completely destroyed at pH 2.5. In purification studies the enzyme has been exposed to pH 13.0 for several minutes with no loss in activity.

*Temperature and penicillinase activity*

Enzymatic destruction of penicillin proceeded best at 37 C within the range tested (table 6). At temperatures below this, the rate of inactivation was substantially retarded, being, however, definitely positive at 0 C. At 49 C the enzyme was destroyed.

TABLE 6  
*Temperature and penicillinase activity*

TEMPERATURE	PENICILLIN* DESTROYED
	<i>per cent</i>
0 C	9
5 C	31
24 C	56
29 C	60
37 C	70
49 C	0

\* Initial concentration of penicillin = 40 units per ml. Enzyme solution concentration = 5 per cent by volume; time = 1 hour.

*pH and penicillinase activity*

The effect of pH on this reaction is given in table 7. Since penicillin is rather unstable in moderately acid and alkaline solutions, a duplicate series was prepared with boiled enzyme. The difference between penicillin content of the boiled and fresh enzyme solutions indicates penicillin destruction due to enzyme action. Penicillinase exhibits maximum activity between pH 6.5 to 8 (table 7), over which range, incidentally, penicillin itself is quite stable (Foster and Wilker, 1943). Thus despite its wide range of pH stability, penicillinase has a narrow pH optimum for its action.

*Inhibitory action of penicillin impurities on penicillinase*

Solutions of two different penicillin preparations were prepared containing 125, 250, 500, 1,000, and 2,000 units per ml. Preparations containing 70 units per mg and 1,650 units per mg (penicillin material) were used. To the above solutions, 1 ml of bacteria-free enzyme broth was added, and the tubes were incubated at 37 C. Portions were removed for assay after  $\frac{1}{2}$ , 1, and 2 hours of incubation; all samples were pasteurized before assay. It is evident (table 8)

that with equivalent concentrations of penicillin the enzyme destroyed much more of pure penicillin than of the impure preparation. Thus it appears that the impurities in the crude preparation have an inhibitory action on penicillinase. The effect is one of rate, since with prolonged incubation destruction of impure penicillin definitely occurs after two hours. This suggests the inhibition is of the competitive type. The effect of crude penicillin preparations on other enzyme systems may have to be reinvestigated in this light.

TABLE 7  
*pH and penicillinase activity*

pH	PENICILLIN* LEFT, UNITS/ML		PENICILLIN DESTROYED  <i>per cent</i>
	Whole enzyme	Boiled enzyme	
4.5	13	15	10
5.0	13	15	12
5.5	16	19	16
6.0	16	21	24
6.5	9	26	66
7.0	8	22	62
7.5	8	21	63
8.0	9	20	56
8.3	13	13	0

\* Initial concentration of penicillin = 25 units per ml. Enzyme solution concentration = 5 per cent by volume; time = 1 hour.

TABLE 8  
*Inhibitory action of impurities on penicillinase*

PENICILLIN ADDED  <i>(units/ml)</i>	UNITS PER ML OF PENICILLIN DECOMPOSED					
	70 unit/mg penicillin			Crystalline penicillin		
	<i>½ hr</i>	<i>1 hr</i>	<i>2 hr</i>	<i>½ hr</i>	<i>1 hr</i>	<i>2 hr</i>
2,000	0	0	500	220	720	1,120
1,000	0	80	260	260	460	580
500	0	210	400	250	310	460
250	0	150	260	145	213	250
125	21	65	125	111	125	125
125 and boiled enzyme	3	16	72	3	33	53

#### *Enzyme poisons*

A number of enzyme poisons were tested for their effect on penicillinase action over a 1-hour period. Table 9 shows that sodium azide, iodoacetic acid, and FeCl<sub>2</sub> were definitely inhibitory to penicillinase activity in the concentrations used (0.01 and 0.1 per cent). The slight effect of sodium fluoride is questionable. Cysteine itself inactivates penicillin (0.1 per cent in the boiled enzyme treatment). The poisons themselves in the concentrations used were found to be

without effect on the assay. Table 10 shows a more detailed experiment on the inhibition of penicillinase action by iodoacetic acid over a 1-hour period. Even the lowest concentration tested,  $M/1860$ , caused an appreciable reduction in destruction of penicillin by the enzyme.

TABLE 9  
*Effect of poisons on penicillinase*

Concentrations of poisons .....	PENICILLIN LEFT, UNITS PER ML			
	Fresh enzyme*		Boiled enzyme	
	0.1%	0.01%	0.1%	0.01%
None.....	0	0	60	60
Na thioglycollate.....	0	0	51	53
Cystine.....	0	0	51	62
Cysteine.....	0	9	8	50
NaF.....	7	5	41	60
NaN <sub>3</sub> .....	39	18	69	51
Iodoacetic acid.....	35	20	62	61
FeCl <sub>2</sub> .....	43	13	60	44

\* 5 per cent by volume.

TABLE 10  
*Inhibition of penicillinase by iodoacetic acid*

IODOACETIC ACID	PENICILLIN* DESTROYED, PER CENT	
	2 per cent enzyme solution	10 per cent enzyme solution
$M/18.6$	0	0
$M/37.2$	0	30
$M/186$	60	56
$M/372$	34	60
$M/744$	30	64
$M/1,860$	26	78
None	96	100

\* Initial concentration of penicillin = 40 units per ml.

#### *Activation of penicillinase*

Penicillinase apparently can be activated by sulfhydryl groups, since penicillin destruction was accelerated by sodium thioglycollate. For example, addition of as little as 0.01 per cent thioglycollate led to a 50 per cent destruction of the penicillin by 2 volume per cent enzyme which alone failed to bring about destruction of any penicillin. When the enzyme solution was used in 10 per cent level, an increase from 64 up to 100 per cent destruction was observed. Higher concentrations of the thioglycollate (0.5 per cent) began to inhibit enzyme action.

*Purification studies*

Table 11 summarizes a number of fractionation experiments directed towards concentrating and purifying penicillinase. Aluminum oxide was a good absorbent of the enzyme. High salt concentrations (M/1.5 phosphate) used as eluants gave practically full recovery in the eluate. Lower concentrations were unsatisfactory. The usual protein salt precipitants failed to precipitate most of the enzyme directly from the bacterial culture fluid, whereas alcohol did. However, the alcohol precipitate was subject to denaturation unless kept cold.

TABLE 11  
*Fractionation experiments on penicillinase*

TREATMENT	RESULTS
1 vol. alcohol 0 C.....	100% in precipitate
2 vol. alcohol 0 C.....	100% in precipitate
4 vol. alcohol 0 C.....	74% in precipitate
1% tannic acid.....	None in precipitate
10% tannic acid.....	None in precipitate
1% lead acetate.....	25% in precipitate
10% lead acetate.....	11% in precipitate
Sat'd $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....	55% in supernatant
Sat'd $(\text{NH}_4)_2\text{SO}_4$ pH 8.0.....	79% in supernatant
Sat'd $(\text{NH}_4)_2\text{SO}_4$ pH 7.0.....	78% in supernatant
Sat'd $(\text{NH}_4)_2\text{SO}_4$ pH 6.0.....	70% in supernatant
1% norite.....	60% absorbed
10% norite.....	100% absorbed
10% norite eluted with 1% ethanol.....	None eluted
10% norite eluted with 1% acetone.....	None eluted
10% norite eluted with 0.1 N HCl.....	1-5% eluted
10% norite eluted with 0.1 N NaOH.....	1-5% eluted
10% norite eluted with M/1.5 phosphate buffer pH 7.2.....	1-5% eluted
1% $\text{Al}_2\text{O}_3$ .....	90% absorbed
1% $\text{Al}_2\text{O}_3$ eluted with M/1.5 phosphate buffer pH 7.2.....	100% eluted
1% $\text{Al}_2\text{O}_3$ eluted with 30% $(\text{NH}_4)_2\text{HPO}_4$ solution.....	100% eluted
Phosphate eluate dialysed against $\text{H}_2\text{O}$ .....	Less than 20% re-covered
$(\text{NH}_4)_2\text{HPO}_4$ eluate treated with equivalent amount of $\text{Ba}(\text{OH})_2$ .....	67% in filtrate

Dialysis experiments repeatedly have shown that some or all of the activity disappears and cannot be recovered even if the residue and dialyzate are combined. Whether the enzyme itself can diffuse to a certain extent or whether an irreversible enzyme-coenzyme change takes place during dialysis is not yet known.

## SUMMARY

The ability to destroy penicillin is a property widespread among bacteria, yeasts, and fungi, with aerobic sporeforming bacteria and certain actinomycoetes being outstanding in this respect. Certain chemical and biological properties

of extracellular penicillinase are described together with experiments relating to its formation by bacteria.

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## MICROBIOLOGICAL ASPECTS OF PENICILLIN

### VIII. PENICILLIN FROM DIFFERENT FUNGI

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Penicillin production now is well known to be a property of different strains of *Penicillium notatum*. Already, however, there are reports which indicate that the antibacterial powers of certain other mold filtrates can be ascribed, in part at least, to their content of true penicillin or of substances which act similarly biologically and chemically in all the properties examined. Although it is impossible to establish the identity of two substances with certainty unless they are available in the pure state, the weight of evidence built up from a number of different lines of approach, chemical and biological, can serve to establish identity or chemical similarity for all practical purposes.

The instances referred to above are (1) the formation of penicillin by *Penicillium chrysogenum* (Smith, 1942), (2) the production of flavicin by *Aspergillus flavus* (Bush and Goth, 1943; McKee and MacPhillamy, 1943; Waksman and Bugie, 1943), and (3) the formation of gigantic acid by *Aspergillus giganteus* (Philpot, 1943) and parasitacin production by *Aspergillus parasiticus* (Cook and Lacey, 1944). *P. chrysogenum* is related to *P. notatum*, and penicillin formation is, therefore, not surprising, although it must be emphasized that, as in the case of *P. notatum*, not all strains within the species group produce penicillin and, moreover, they may vary greatly in this property (Clutterbuck, Lovell, and Raistrick, 1932; Foster, Woodruff, and McDaniel, 1943). It seems advisable that before naming newly discovered antibiotic substances they be proved dissimilar to those included in the rapidly growing list of substances already known. This is especially true now that more than one organism is known to produce the same antibiotic substance. For example, penicillic acid is produced by *Penicillium puberulum* and *Penicillium cyclopium* (Oxford, Raistrick, and Smith, 1942) and *Aspergillus ochraceus*, *Penicillium thomii*, and *Penicillium suavolens* (Karow, Woodruff, and Foster, 1944); citrinin by *Penicillium citrinum* (Hetherington and Raistrick, 1931) and *Aspergillus candidus* (Timonin, 1942); and the substance variously called clavacin, claviformin, and patulin is produced by *Aspergillus claviforme* (Bergel *et al.*, 1943), *Aspergillus clavatus* (Hooper *et al.*, 1944; Waksman *et al.*, 1942; Wiesner, 1942), *Penicillium patulum* (Raistrick *et al.*, 1943), and *Gymnoascus* sp. and *Penicillium melinii* (Karow and Foster, 1944).

The rather widespread occurrence of penicillin-destroying enzyme(s) in diverse microbial species (Abraham and Chain, 1940; Woodruff and Foster, 1944) which presumably are never exposed to penicillin in nature induces the speculation

that penicillin or penicillinlike substances exist in and play a rôle in the metabolism of organisms other than *P. notatum*.

This paper provides further evidence for a more general distribution of penicillin systems by demonstrating the formation of penicillin, or substances indistinguishable from it, in cultures of five more fungi, namely, *Aspergillus oryzae* TP, *Aspergillus nidulans*, *Aspergillus niger* YW, *Aspergillus flavipes*, and *Penicillium citreo-roseum*. The experimental evidence is in six categories: (1) solubility characteristics, (2) pH stabilities, (3) temperature stabilities, (4) antibacterial spectra, (5) penicillinase inactivation, and (6) *in vivo* therapeutic efficacy (one case). The amounts of penicillin produced in each case are small compared to good strains of *P. notatum*; nevertheless, it has been possible to effect satisfactory concentrations. All of the above-named five fungi were furnished through the courtesy of Dr. Charles Thom. More than one strain of each species produced antibacterial filtrates under these conditions; however, only a single strain was selected for detailed study.

#### EXPERIMENTAL

*Production of active substances.* Each of the organisms (*A. flavipes* excepted) was tested for its ability to produce antibacterial activity in surface cultures in four different media: (a) Czapek-Dox, (b) Czapek-Dox with brown sugar instead of glucose, (c) peptone glucose, (d) corn steep medium (Czapek-Dox plus 3 per cent corn steep liquor). Incubation was at 25 C unless otherwise noted. Significant activity was produced only in the corn steep medium, which greatly promoted growth in each case. The highest activities obtained, expressed as the reciprocal of the dilution required for inhibition of *Staphylococcus aureus* H in nutrient broth, were: *A. niger* YW, 256; *A. nidulans*, 128; *A. oryzae* TP, 128; *P. citreo-roseum*, 128. Occasionally there was a titer of eight in some of the other media.

*Solvent extraction and concentration of the active agents.* Active corn steep broth was cooled to 5 C, acidified to pH 2.0 with HCl, and extracted with 2 volumes of ether in a separatory funnel. The ether layer was quickly treated with  $\frac{1}{10}$  its volume of M/50 phosphate buffer at pH 7.0 into which the major portion of the penicillin passed. The cooled buffer was in turn acidified to pH 2.0 and extracted with 2 volumes of ether from which the penicillin was again extracted with a 0.1 N  $\text{Na}_2\text{CO}_3$  solution in a volume  $\frac{1}{10}$  that of the original broth. The final pH of the  $\text{Na}_2\text{CO}_3$  extract was about 7.3. Table 1 shows the activities of the aqueous stages of the concentration procedure. The parallelism with penicillin produced by *P. notatum* is obvious. Usually about 50 per cent recovery was obtained, although, in the case of *A. oryzae*, recovery was 100 per cent within the 30 per cent latitude of the dilution assay method. The solubility characteristics of authentic penicillin were identical under the above conditions.

The slight activity produced by *A. oryzae* TP in Czapek-Dox medium behaved differently from that produced by the same organism in corn steep media. The antibacterial fraction was concentrated and isolated as a pure crystalline

substance which was identified as kojic acid (5-hydroxy-2-hydroxymethyl- $\gamma$ -pyrone). This substance was first isolated from cultures of *A. oryzae* by Yabuta (1912) who later established its structure (1924). The production of kojic acid by different species of *Aspergillus* was studied by Birkinshaw *et al.* (1931). Yabuta noted that bacterial growth is eliminated in the presence of more than 0.5 per cent of the acid, and Friedmann (1934) showed that kojic acid is toxic for rabbits at 150 mg per kg.

The last column in table 4 shows the amount of sodium kojate inhibiting pure cultures of various bacteria. There was unexpected specificity for the gram-negative bacteria as compared to the gram-positive ones as a whole. Invariably the group of gram-positive organisms listed has proved to be at least

TABLE 1

*Extraction and concentration of antibacterial activity produced by various fungi*

ORGANISM	TREATMENT	DILUTION INHIBITING <i>S. AUREUS</i>
<i>A. oryzae</i> TP	Original broth	150
	M/50 buffer	1,740
	Na <sub>2</sub> CO <sub>3</sub> extract	15,000
<i>A. nidulans</i>	Original broth	100
	M/50 buffer	780
	Na <sub>2</sub> CO <sub>3</sub> extract	5,000
<i>A. niger</i>	Original broth	150
	M/50 buffer	800
	Na <sub>2</sub> CO <sub>3</sub> extract	6,250
<i>P. citreo-roseum</i>	Original broth	40
	M/50 buffer	400
	Na <sub>2</sub> CO <sub>3</sub> extract	600
<i>P. notatum</i>	Original broth	1,600
	M/50 buffer	13,600
	Na <sub>2</sub> CO <sub>3</sub> extract	147,000

as sensitive to numerous antibacterial substances as the gram-negative ones, and generally much more so. This reverse effect with kojic acid is, therefore, of special interest.

*pH stability of the active fractions.* The stability of the active agent in concentrates was determined by allowing the concentrates to stand at room temperature for 2 hours at different pH values, neutralizing to pH 7.0, and reassaying (table 2).

At pH 4.0 or less the activity was rapidly destroyed in the concentrates, a result which was comparable to the findings on the penicillin control. (See also Foster and Wilker, 1943.)

*Thermostability of the active fractions.* This was carried out at pH 7.5 for 2 hours at 60 C and 100 C.

The heat stabilities (table 3) were comparable to that of penicillin. Two hours at 100 C destroyed all of the activity in every case, and at 60 C, with the exception of *A. oryzae* in which case an assay discrepancy exists, the activities

were partially destroyed. In the case of true penicillin the concentration was so high that despite a large loss some was still left at 100 C.

*Antibacterial spectra of the active fractions.* The ability of the different preparations to inhibit a variety of different gram-positive and gram-negative bacteria was tested by the plate streak method on brain heart infusion agar containing graded levels of the test substances. The dilutions differed by a threefold

TABLE 2  
*pH stability of antibacterial agents*

pH	RESIDUAL PENICILLIN ACTIVITY				
	<i>A. oryzae</i> TP	<i>A. nidulans</i>	<i>A. niger</i>	<i>P. citreo-roseum</i>	<i>P. notatum</i>
2.0	0*	0*	0†	0†	0†
3.0	—	—	1,700	—	16,000
4.0	27	0	3,750	10	76,000
5.0	—	—	7,500	40	124,000
6.0	67	38	7,500	—	160,000
7.0	—	—	—	—	124,000
8.0	57	27	—	40	148,000
Original solution	75	50	7,500	40	147,000

\* Oxford units per ml.

† *S. aureus* dilution.

TABLE 3  
*Thermostability of antibacterial agents*

ORGANISM	ROOM TEMPERATURE		60 C	100 C
<i>A. oryzae</i> TP	Exp't. A*	75	84	0
	Exp't. B	8,000	1,460	0
<i>A. nidulans</i> *		50	40	0
<i>A. niger</i>	Exp't. A	7,500	1,700	0
	Exp't. B	15,600	11,200	0
<i>P. citreo-roseum</i> *		40	40	0
<i>P. notatum</i>		147,000	97,000	44,000

\* Oxford units per ml. All other values are expressed as dilutions inhibiting *S. aureus*.

factor. An authentic penicillin preparation was included for comparative purposes. The results (table 4) are expressed as the ratios of the amount of active agents required to inhibit the various test bacteria compared to *Staphylococcus aureus* H taken as unity.

It is evident that the substances derived from the five different fungi follow fairly closely the same pattern in their antibacterial spectra. Since the different

new agents were separately compared with authentic penicillin, the corresponding penicillin controls are given in each case. Although the absolute values were not always identical for a given test bacterium, they are well within the same order of magnitude, and the parallelism with true penicillin is clear. Those instances in which the figures are "greater than" represent concentrations which were the highest tested at that time.

One organism in particular seemed to be out of line in repeated experiments. *Bacillus cereus* was 300 times more sensitive to *P. citreo-roseum* material than to authentic penicillin. There is evidence for the existence of a second substance

TABLE 4

Comparative inhibition\* of different bacteria by active substances derived from various fungi

TEST BACTERIA	SOURCE OF ACTIVE SUBSTANCES							Kojic acid mg/ml
	Authen- tic peni- cillin	<i>A. oryzae</i> TP	<i>A. nidu- lans</i>	Authentic penicillin	<i>A. niger</i>	Authen- tic peni- cillin	<i>P. citreo- roseum</i>	
<i>Staphylococcus aureus</i> H ..	1	1	1	1	1	1	1	0.8
<i>Streptococcus pyogenes</i> .	100	100	100	100	100	100	100	>1.0
<i>Streptococcus pyogenes</i> MIT	100	100	100	33	100	100	30	>1.0
<i>Streptococcus pyogenes</i> M...	100	100	100	100	100	100	30	>1.0
<i>Micrococcus</i> MY . . . . .	1	10	1	1	3	3	3	>1.0
<i>Micrococcus lysodeikticus</i> ..	3	10	1	0.3	0.3	1	1	0.4
<i>Staphylococcus albus</i>	1	1	1	1	3	1	1	>1.0
<i>Staphylococcus aureus</i> FDA	1	1	1	1	1	1	1	1.0
<i>Pneumococcus</i> III . . . . .	33	100	33	100	100	100	30	>1.0
<i>Pseudomonas aeruginosa</i> . .	>3,300	>300	>100	>3,300	>100	>3,000	>5,883	0.8
<i>Escherichia coli</i> . . . . .	3,300	>300	>100	3,300	>100	>3,000	>5,883	0.6
<i>Eberthella typhosa</i>	330	>300	>100	330	1000	300	100	0.6
<i>Salmonella paratyphi</i>	10	10	10	10	10	10	3	>1.0
<i>Salmonella schottmuelleri</i>	33	100	100	33	100	100	30	0.6
<i>Bacillus subtilis</i> .....	3	10	3	1	3	1	1	>1.0
<i>Bacillus cereus</i> . . . . .	>3,300	>300	>100	1000	1000	3000	10	1.0
<i>Bacillus megatherium</i> . . . . .	3	100	3	10	10	10	10	1.0
<i>Pasteurella</i> sp. . . . .	100	>300	100	33	330	100	30	0.6

\* *Staphylococcus aureus* as unity.

in the *P. citreo-roseum* preparation which is particularly active against *B. cereus*. This is under investigation.

*Inactivation by bacterial penicillinase.* The well-known specificity of enzymes frequently facilitates establishment of the identity or similarity of unknown substances with one of known properties by comparing the action of the enzyme on the respective substrates. Penicillinase was discovered by Abraham and Chain (1940), and certain of its properties were described in detail by Woodruff and Foster (1944).

Culture filtrates and concentrates of the antibiotic agents from the five dif-

ferent molds were treated with a cell-free preparation of penicillinase derived from an unidentified bacterium (culture 164). (See Woodruff and Foster, 1944.) In each case suitable boiled enzyme controls were run. The enzyme was allowed to act for 1 hour at 37 C, followed by pasteurization at 60 C for 30 minutes to halt its action. Pasteurization has only a small effect on the penicillin content of solutions (Foster and Wilker, 1943; Woodruff and Foster, 1944). The results of these experiments are recorded in table 5. The *Bacillus subtilis* modification (Foster and Woodruff, 1943, 1944) of the original Oxford cup assay (Abraham *et al.*, 1941) was used.

TABLE 5

*Penicillinase destruction of antibacterial activity of agents derived from different fungi*

	OXFORD UNITS/ML
<i>P. notatum</i> concentrate + boiled enzyme.....	29.5
<i>P. notatum</i> concentrate + fresh enzyme.....	0
<i>A. oryzae</i> TP culture filtrate + boiled enzyme. ....	0.5
<i>A. oryzae</i> TP culture filtrate + fresh enzyme ..	0
<i>A. oryzae</i> TP concentrate + boiled enzyme.....	94
<i>A. oryzae</i> TP concentrate + fresh enzyme.....	0
<i>A. nidulans</i> concentrate + boiled enzyme ..	26.5
<i>A. nidulans</i> concentrate + fresh enzyme.....	0
<i>A. niger</i> broth + boiled enzyme .....	2
<i>A. niger</i> broth + fresh enzyme. ....	0
<i>A. niger</i> concentrate + boiled enzyme....	156
<i>A. niger</i> concentrate + fresh enzyme ..	0
<i>P. citreo-roseum</i> broth + boiled enzyme. ....	0.4
<i>P. citreo-roseum</i> broth + fresh enzyme. ....	0
<i>P. citreo-roseum</i> concentrate + boiled enzyme. ....	24
<i>P. citreo-roseum</i> concentrate + fresh enzyme.....	0
<i>A. flavipes</i> culture filtrate + boiled enzyme....	1.9
<i>A. flavipes</i> culture filtrate + fresh enzyme..	0

The complete destruction enzymatically of the antibacterial activity of all the various solutions listed in table 1 is further presumptive evidence of their respective similarities with penicillin.

After this paper was written a communication by White (1943) appeared reporting antibacterial activity in culture filtrates of *Aspergillus flavipes* (no. 175-4303.46). Using this same strain, we found the antibacterial activity it produced in 2 per cent tryptone medium (White, 1943) was completely destroyed by a 30-minute incubation with penicillinase. It seems probable that the antibiotic substance was penicillin or penicillinlike.

*In vivo* protection efficacy test. The ability of penicillin to afford protection against lethal doses of virulent bacteria is, on the basis of *in vitro* antibacterial potency, distinctive enough to aid in the identification of substances suspected to be penicillin or penicillinlike. This was employed in the case of one of the fungi used above, namely, *A. niger*. Several liters of culture filtrate from this

mold were extracted in a manner similar to that described by Abraham *et al.* (1941). The dry end product, the sodium salt, closely resembled similarly extracted preparations of true penicillin from *P. notatum*. It had the typical bright orange-yellow color and contained 40 Oxford units per mg. A portion dissolved in water and treated with cell-free penicillinase was completely inactivated very rapidly.

Mice were infected intraperitoneally with 10,000 lethal doses of *Diplococcus pneumoniae* Type I (no. 37) by Dr. H. Robinson of the Merck Institute. Treatment was initiated immediately after the bacterial inoculation and was repeated afterwards at 6-hour intervals over a 24-hour period. A total of 792 Oxford units was given to each mouse in the 24-hour period. Observations were then made over the following 12 days. For comparison, a similar group of infected mice was treated with a standard sample of authentic penicillin (table 6). The *A. niger* preparation afforded complete protection in doses equal to those of authentic penicillin also providing complete protection.

TABLE 6

*In vivo efficacy of preparation from A. niger*

Strain: *Diplococcus pneumoniae* Type I (no. 37)

Age: 6-hour culture

Infection: 0.5 ml of  $10^6$  dilution of culture equivalent to 10,000 lethal doses

Therapy: 198 Oxford units every 6 hours for 24 hours by subcutaneous injection

SAMPLE	NO. OF MICE	NO. OF MICE SURVIVING	TIME IN DAYS
<i>A. niger</i> . . . . .	3	3	12
Authentic penicillin . . . . .	3	3	12
Untreated controls . . . . .	12	0	1

*The effect of temperature on penicillin production by various fungi.* All previous reports dealing with penicillin production by *P. notatum* state specifically that the optimum temperature for the process is about 25 C. Bush and Goth (1943) cultivated *A. flavus* at 35 to 37 C and obtained flavicin (penicillin?), but the production at other temperatures was not reported. Fleming (1929) and Abraham *et al.* (1941) stated that *P. notatum* does not grow at 37 C.

A systematic study of the temperature influence was made with the five organisms employed. From table 7 it is evident that different fungi may have different optimum temperatures for growth and for penicillin production. *A. niger* and *A. nidulans* were like *P. notatum* in that their optimum temperature for penicillin production was 25 C. *A. niger*, also like *P. notatum*, did not grow at 37 C, whereas *A. nidulans* grew well at that temperature but made no penicillin. *A. oryzae* TP, on the other hand, grew best at 37 C, and this temperature was most favorable for penicillin production both from the standpoint of rate of formation and total produced. That the active substance produced by *A. oryzae* TP at this higher temperature actually was penicillin seems indicated by



penicillinase destruction in one hour of the full activity, as shown in the following tabulation:

	OXFORD UNITS PER ML	
	6-day culture	7-day culture
Broth and boiled enzyme .....	2.0	1.5
Broth and fresh enzyme .....	0	0

The effect of increased temperature on the growth of *A. oryzae* was very marked. A thick, wrinkled pellicle was formed in 3 days at 37 C, but at 25 C the pellicle was still very thin.

*Strain selection.* The well-known differences in the ability to produce penicillin of various strains of *P. notatum* and, as well, the differences in the progeny

TABLE 7  
*Temperature and penicillin production by various fungi*  
(Corn steep liquor medium)

INCUBATION TIME	A. NIGER			A. ORYZAE TP			A. NIDULANS			P. CITREO-ROSEUM			P. NOTATUM		
days	25 C	30 C	37 C	25 C	30 C	37 C	25 C	30 C	37 C	25 C	30 C	37 C	25 C	30 C	37 C
5	0	0.5	—	0	0.6	1.3	0	0	0				>4.0	4.0	—
6	2.1	0.8	—	0	1.2	2.2	0	0	0	0.1	0	—	13.5	>8.0	—
7	2.5	1.1	—	0	1.2	2.0							17.8	7.0	—
8	3.2	—	—	0.7	1.6	2.1	0.9	0	0	0.2	0.1	—	22.8	11.6	—
9	4.9	0.8	—	1.2	1.6		0.8	0.2	0				32.0	16.0	—
10	4.6	1.6	—	0.5	1.4	2.0	0.4	0.3	0	1.0	0.8	—	16.0	6.7	—
12	4.8	0.4	—	1.2	1.2	0.9	0.9	0.2	0	2.0	0	—	31.0	8.9	—

Figures are Oxford units per ml. (—) = no growth.

of a single spore colony (Clutterbuck, Lovell, and Raistrick, 1932; Foster, Woodruff, and McDaniel, 1943) made it reasonable to expect that the low potencies of mold filtrates could be increased by strain selection. *A. oryzae* TP was plated out on peptone glucose agar and eleven substrains isolated and compared with the parent strain for penicillin production in regular corn steep medium. The results (experiment A, table 8) show clearly that two of the substrains were definitely superior to the other substrains and to the parent itself. At all stages of incubation this superiority prevailed. A similar experiment with 11 substrains of *A. niger* YW failed to reveal differences between the substrains and the parent. Experiment B (table 8) shows another comparison between the two best strains from experiment A, namely, nos. 1 and 2, and two poor strains, nos. 6 and 11, and the parent strain from which all four were derived. Each was tested in three different media: corn-steep brown sugar, corn-steep Czapek-Dox, and the brown sugar medium of Hobby, Meyer, and Chaffee (1942). The activity produced in the last-named medium was virtually negli-

TABLE 8  
*Penicillin production by substrains of A. oryzae TP*  
*Experiment A*

STRAIN NO.	OXFORD UNITS PER ML				
	4 days	5 days	6 days	9 days	10 days
Parent	0.6	1.4	1.6	0.9	1.1
1	>2	>4	6.4	4.6	2.5
2	>2	>4	7.6	6.2	3.7
3	1.0	1.5	1.5	1.9	1.5
4	0.8	0.9	1.2	1.6	1.4
5	0.6	1.0	1.1	2.1	1.6
6	1.3	2.1	1.5	1.2	1.6
7	0.8	1.1	1.2	1.8	1.6
8	0.7	1.0	1.2	1.8	1.6
9	0.9	0.9	1.5	1.6	0.6
10	0.7	1.0	1.6	0	0
11	1.3	1.6	1.7	0.8	1.7

*Experiment B*

STRAIN NO	CORN STEEP MEDIUM WITH	OXFORD UNITS PER ML			
		3 days	4 days	5 days	6 days
1	Brown sugar	3.1	15.0	16.2	8.0
	Czapek-Dox	2.7	6.5	7.3	4.8
2	Brown sugar	7.0	16.0	14.2	8.4
	Czapek-Dox	2.8	6.3	5.7	5.6
6	Brown sugar	1.0	3.1	3.3	2.8
	Czapek-Dox	0.7	1.3	1.4	1.1
11	Brown sugar	1.9	3.7	4.9	3.2
	Czapek-Dox	1.0	1.7	1.7	1.4
Parent	Brown sugar	1.8	4.1	4.7	3.2
	Czapek-Dox	0.9	1.3	1.6	1.6

*Experiment C*

STRAIN NO.	OXFORD UNITS PER ML			
	3 days	4 days	5 days	7 days
2-1	7.7	13.4	18.2	7.2
2-2	8.5	16.0	17.8	6.8
2-3	6.9	15.0	15.6	6.2
2-4	11.6	25.0	14.6	6.4
2-5	9.0	21.8	15.6	7.8
2-6	4.3	11.8	14.2	6.2
2-7	4.7	17.2	15.6	7.8
2-8	7.3	16.8	16.4	6.2
2-9	7.3	15.8	17.0	5.6
2-10	4.5	15.0	17.0	5.2
2-11	6.9	16.8	18.8	6.4
2-12	4.8	11.8	17.0	7.0
Parent, strain 2	3.8	13.6	14.0	7.2

gible in all cases and is not, therefore, reported in the table. The results confirm those of experiment A in bringing out the decided differences between strains 1 and 2 and the other three. In both media there was a 3- to 5-fold superiority, the corn-steep brown-sugar medium being better than corn-steep Czapek-Dox for all the strains.

The selection process was continued one step further. Strain 2 was replated and 12 single colony isolates picked and tested. The results in experiment C, table 8, show that 2 of these 12 substrains were decidedly superior to the immediate parent strain. At peak production strain 2-4 was twice as effective as the parent.

It has, therefore, been possible to effect a 6- to 10-fold increase in penicillin-producing powers by a two-stage strain selection procedure. Possibly, continuation of this procedure could lead to the isolation of still better strains.

#### SUMMARY

Antibacterial substances present in culture filtrates of *Aspergillus niger* YW, *Aspergillus nidulans*, *Aspergillus oryzae* TP, *Aspergillus flavipes* and *Penicillium citreo-roseum* are either identical with, or closely related to, authentic penicillin produced by *Penicillium notatum*. This was shown by solubility properties, thermostability, pH stability, antibacterial spectra against 18 different bacteria, destruction by the enzyme penicillinase, and (in one instance) by efficacy in protecting animals against a lethal bacterial infection. Substrains superior in penicillin-producing ability to the parent culture of *A. oryzae* TP were isolated. *A. oryzae* TP grew and produced penicillin best at 37 C; with the other fungi the optimum was 25 C. *A. nidulans* grew at 37 C but produced no penicillin. *P. notatum*, *A. niger*, and *P. citreo-roseum* did not grow at 37 C. Kojic acid was more effective in inhibiting the gram-negative bacteria tested than the gram-positive ones.

*Addendum:* While this paper was in press penicillinlike substances were shown to occur in cultures of 5 species of *Penicillium* different from any mentioned in this paper, (Florey, Heatley, Jennings, and Williams, *Nature*, **154**, 268, 1944).

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# THE UTILIZATION OF $\beta$ -ALANINE AND PANTOTHENIC ACID BY YEASTS<sup>1</sup>

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In 1936  $\beta$ -alanine was shown to be a nutritive for yeast (Williams and Rohrmann); later it was found to constitute part of the pantothenic acid molecule (Weinstock *et al.*, 1939). Pantothenic acid is required for the growth of many yeasts (Williams, Eakin, and Snell, 1940; Leonian and Lilly, 1942; Lochhead and Landerkin, 1942) and may be replaced in some cases by  $\beta$ -alanine. Wieland and Möller (1942) have studied the influence of the ammonium ion on the utilization of  $\beta$ -alanine by yeast and Nielsen and coworker (1941, 1943) have experimented with yeast growth stimulation by amino acids. A few of the amino acids promote the growth of yeast when present in large amounts but none in the low concentration required by  $\beta$ -alanine.

Lactic acid bacteria cannot utilize  $\beta$ -alanine to replace pantothenic acid even when the lactone moiety of the pantothenic acid molecule is also supplied (Snell, Strong, and Peterson, 1939; Cheldelin, Hoag, and Sarett, 1945). The diphtheria bacillus is the only organism besides yeasts which is known to respond to  $\beta$ -alanine (Mueller and Cohen, 1937).  $\beta$ -alanine cannot replace pantothenic acid for maintenance and growth of rats (György, Poling, and Subbarow, 1939) or chicks (Woolley, Waisman, and Elvehjem, 1939).

The present work is a comparison of the utilization of  $\beta$ -alanine and pantothenic acid in synthetic medium by several strains of *Saccharomyces cerevisiae* and one of *Saccharomyces carlsbergensis*. This work was originally undertaken in an attempt to develop a method for assay of  $\beta$ -alanine. The data in this paper indicate that the yeasts studied cannot be used under these conditions for the measurement of  $\beta$ -alanine because of the inhibition by amino acids and natural extracts.

## EXPERIMENTAL

The growth factors and test substances (at pH 4.8) are measured into 20 x 150 mm, lipless, pyrex test tubes, diluted to a total volume of 2 ml, and 5 ml of the medium shown in table 1 are added to each tube. The medium used is based upon that designed by Snell *et al.* (1940) for biotin assay. The sucrose is replaced by glucose,  $\beta$ -alanine is omitted, other B vitamins are added, and the vitamin concentrations are increased as suggested by Leonian and Lilly (1943).

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The tubes containing the samples and medium are steamed without plugging and inoculated when cool.

The yeasts are grown on molasses agar slants<sup>2</sup> for 24 hours at 30 C and then kept in a refrigerator. For an inoculum, a loopful of yeast from a freshly grown slant is suspended in a tube of sterile medium and the yeast concentration determined turbidimetrically with the aid of a standardized calibration curve. A measured amount of this suspension is transferred to a flask containing a known volume of sterile medium so that the concentration of moist yeast is 2.4 mg per 100 ml. The test is then seeded with 1 ml added to each tube to give a final volume of 8 ml and a yeast concentration of 0.3 mg per 100 ml. The tubes are incubated at 30 C for 16 to 18 hours and the turbidity measured photoelectrometrically (5400 Å filter) after cooling in a refrigerator and adding 2 ml of a saturated solution of *p*-chlorothymol per tube. The results are given in terms

TABLE 1  
*Basal medium*

Glucose . . . . .	20 g
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> . . . . .	3 g
KH <sub>2</sub> PO <sub>4</sub> . . . . .	2 g
<i>L</i> -Aspartic acid . . . . .	0.1 g
Salt solutions* 1 and 2 . . . . .	1 ml each
Adenine sulfate . . . . .	20 mg
Inositol . . . . .	15 mg
Thiamin, riboflavin, nicotinic acid, pyridoxine and <i>p</i> -aminobenzoic acid	200 µg each
Biotin . . . . .	1 µg
Folic acid† . . . . .	2 µg
Distilled water to 1 liter; pH 4.8 to 5.0	

\* Salt solution 1 contains 0.25 g CaCl<sub>2</sub>, 0.5 mg FeCl<sub>3</sub>, and 1.0 mg each of MnCl<sub>2</sub> and TiCl<sub>3</sub> per ml; solution 2 contains 0.25 g MgSO<sub>4</sub>·7 H<sub>2</sub>O, 1 mg each of ZnSO<sub>4</sub> and H<sub>3</sub>BO<sub>3</sub>, and 0.1 mg each of CuSO<sub>4</sub>·5 H<sub>2</sub>O and KI per ml (Snell, Eakin, and Williams, 1940).

† A folic acid concentrate was kindly furnished by Dr. R. J. Williams of the University of Texas, and is used here in terms of 40,000 potency.

of optical density which is equal to log 100 minus log per cent transmission (2-log G).

## RESULTS

In the presence of various levels of  $\beta$ -alanine, the effects of 1 mg taurine, 2 mg pantoyltaurine<sup>3</sup> (the sulfonic acid analog of pantothenic acid), 2 mg asparagine, and 50 µg of the lactone<sup>4</sup> portion of the pantothenic acid molecule per tube have been measured for 17 yeasts. In the presence of pantothenic acid only

<sup>2</sup> The molasses agar has the following composition: 4 per cent molasses, 0.12 per cent (NH<sub>4</sub>)<sub>2</sub>H<sub>2</sub>PO<sub>4</sub>, and 2 per cent agar.

<sup>3</sup> We are indebted to Dr. D. W. Woolley, Rockefeller Institute for Medical Research, for a gift of pantoyltaurine, N-( $\alpha,\gamma$ -dihydroxy- $\beta,\beta$ -dimethylbutyryl) taurine.

<sup>4</sup> In the present communication lactone refers to the cleavage product of pantothenic acid, *l*- $\alpha$ -hydroxy- $\beta,\beta$ -dimethyl- $\gamma$ -butyrolactone. This compound was kindly supplied by Dr. J. C. Keresztesy of Merck & Co., Inc., Rahway, N. J.

TABLE 2  
Turbidimetric growth response of 17 yeasts\* to  $\beta$ -alanine and calcium pantothenate

CONTENTS PER TUBE	BER- GUNDY WINE	F. B.	GE- BRIDER MAVER	#1 HANSEN	LASH MILLER	OLD PROC- ES	RASSE M (Kogl)	S. C.	XR	578	2190	2331	2335	2504	4097	4098	S CARLS. 4228
$\mu$ g																	
$\beta$ -alanine																	
0.0	0.020	0.015	0.025	0.020	0.020	0.015	0.005	0.015	0.010	0.015	0.000	0.010	0.030	0.150	0.020	0.025	0.015
0.2	0.090	0.035	0.465	0.035	0.195	0.025	0.080	0.130	0.020	0.025	0.000	0.015	0.250	0.395	0.055	0.045	0.130
0.5	0.340	0.135	0.600	0.095	0.400	0.050	0.370	0.400	0.040	0.030	0.002	0.025	0.665	0.530	0.165	0.085	0.400
2.0	0.405	0.630	0.595	0.245	0.620	0.115	0.560	0.530	0.470	0.415	0.003	0.055	0.705	0.455	0.260	0.680	0.490
5.0	0.405	0.635	0.620	0.275	0.630	0.500	0.570	0.550	0.600	0.490	0.001	0.370	0.700	0.450	0.185	0.710	0.550
Calcium pantothenate																	
0.1	0.300	0.355	0.275	0.075	0.305	0.230	0.120	0.265	0.085	0.120	0.040	0.130	0.430	0.450	0.225	0.380	0.265
0.2	0.420	0.570	0.470	0.250	0.560	0.470	0.350	0.480	0.275	0.390	0.170	0.335	0.670	0.490	0.300	0.640	0.480
0.5	0.410	0.610	0.580	0.450	0.600	0.520	0.600	0.550	0.610	0.575	0.085	0.570	0.670	0.490	0.310	0.730	0.530
2.0	0.420	0.620	0.620	0.440	0.620	0.540	0.610	0.555	0.610	0.585	0.065	0.600	0.730	0.510	0.315	0.720	0.555
0.1 + PT†	0.125	0.070	0.060	0.025	0.070	0.040	0.010	0.080	0.020	0.080	0.015	0.030	0.095	0.315	0.080	0.060	0.080
0.2 + PT†	0.270	0.240	0.190	0.050	0.205	0.140	0.035	0.185	0.040	0.090	0.010	0.085	0.220	0.435	0.165	0.150	0.185
0.1 + A‡	0.290	0.390	0.310	0.080	0.300	0.250	0.135	0.330	0.080	0.190	0.050	0.165	0.485	0.550	0.225	0.420	0.330
0.2 + A‡	0.450	0.630	0.500	0.290	0.570	0.600	0.350	0.495	0.330	0.440	0.185	0.380	0.730	0.520	0.300	0.750	0.495
2.0 + A‡	0.440	0.660	0.710	0.490	0.660	0.640	0.680	0.600	0.690	0.680	0.140	0.650	0.790	0.540	0.310	0.800	0.605
2.0 + Y§	0.440	0.780	0.890	0.640	0.810	0.800	0.780	0.650	0.800	0.800	0.500	0.680	0.880	0.600	0.400	0.890	0.670

\* The first 16 yeasts are strains of *Saccharomyces cerevisiae* and were obtained through the kindness of Drs. R. J. Williams and E. E. Snell of the University of Texas. F. B. was isolated from a cake of Fleischmann's yeast and #1 Hansen was the yeast Wildiers used for bios (1901). No. 2190 is from the National Collection of cultures (London) and the other numbered strains refer to the American Type Culture Collection. *Saccharomyces carlsbergensis*, strain 4228, was generously given to us by Dr. C. N. Frey of the Fleischmann Laboratories.

† 2 mg of pantoic acid added per tube.

‡ 2 mg of asparagine added per tube.

§ 5 mg of Difco yeast extract added per tube.



asparagine and pantoyltaurine have been tested. Table 2, however, shows only the growth response of the 17 yeasts to  $\beta$ -alanine and pantothenic acid, and the effect of asparagine and pantoyltaurine on the growth with pantothenic acid. The substances tested in the presence of  $\beta$ -alanine give uniform results for all yeasts except one, and the figures are omitted from the table to conserve space.

Lactone does not alter the response of any of the yeasts to  $\beta$ -alanine. Taurine and pantoyltaurine inhibit the growth with  $\beta$ -alanine for the Lash Miller strain (85 to 90 per cent inhibition of 0.2 or 0.5  $\mu$ g  $\beta$ -alanine with 1 mg taurine or 2 mg pantothenic acid) but are without effect on the  $\beta$ -alanine results of all the others. On the other hand, the inhibition by pantoyltaurine of the utilization of pantothenic acid varies in degree for all the yeasts (table 2). These results agree with the findings of Snell (1941) who observed no inhibition of growth with  $\beta$ -alanine by either taurine or pantoyltaurine but competitive inhibition of pantothenic acid by pantoyltaurine. The inclusion of small amounts of  $\beta$ -alanine lessens the inhibitory effect of pantoyltaurine on pantothenic acid.

In the presence of low concentrations of  $\beta$ -alanine (0.2 or 0.5  $\mu$ g per tube) 2 mg of asparagine strongly curtails the growth of all but 2 of the yeasts, allowing little more growth than is obtained in the blank tubes. Exceptions are strains Lash Miller and 2504, which are inhibited only 20 to 30 per cent under the same conditions. Weinstock *et al.* (1939) reported this inhibition of  $\beta$ -alanine by asparagine and it has been recently used by Atkin *et al.* (1944) in a yeast pantothenic acid method to suppress any  $\beta$ -alanine effects. In the presence of excess  $\beta$ -alanine, 5  $\mu$ g per tube, asparagine either has no effect or is stimulatory for the yeasts. This effect is about the same as the stimulation shown in table 2 upon the addition of asparagine to 2  $\mu$ g pantothenic acid. With the smaller concentrations of pantothenic acid, asparagine does not inhibit and in some cases is stimulatory.

All of the yeasts grow faster and more heavily in the presence of 5 mg Difco yeast extract than in the synthetic medium to which only excess pantothenic acid has been added. The difference is least for Burgundy wine yeast and greatest for strain 2190. Leonian and Lilly (1943) find that in the presence of large amounts of synthetic vitamins, added yeast extract does not increase the growth obtained with 8 yeasts in 72 hours. This is not found after 18 hours for the 17 yeasts tested here.

The yeasts also differ in their total growth in this short time, and in their relative ability to utilize  $\beta$ -alanine and pantothenic acid, with the medium employed. For strain 2190,  $\beta$ -alanine cannot replace pantothenic acid for growth even if the lactone is supplied. Strain Gebrüder Mayer is most sensitive to  $\beta$ -alanine, showing in low concentrations about one third the activity of pantothenic acid on a molecular basis, and equal growth in the presence of excess  $\beta$ -alanine or pantothenic acid. The following yeasts also show equal growth with 5  $\mu$ g  $\beta$ -alanine or 2  $\mu$ g pantothenic acid but with decreasing sensitivity to  $\beta$ -alanine: 2504, 2335, Lash Miller, *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis* 4228, Burgundy wine, Rasse M., FB, 4098, old process, and XR. The remaining yeasts cannot utilize 5  $\mu$ g  $\beta$ -alanine so well as 2  $\mu$ g pantothenic

acid: No. 1 Hansen, 578, 2331, and 4097. Growth of 2504 and 4097 is inhibited by excess  $\beta$ -alanine, and that of 2190 by excess pantothenic acid.

*Study of the Gebrüder Mayer yeast.* The following experiments show the effects of the composition of the medium and of the addition of other substances on the utilization of  $\beta$ -alanine and pantothenic acid by Gebrüder Mayer yeast, which is most sensitive to  $\beta$ -alanine.

In the present experiments, the addition of extra inositol and biotin to the medium of Snell *et al.* (1940) is stimulatory for the 18-hour growth of the Gebrüder Mayer strain. This is in agreement with the observations of Leonian and Lilly (1943). Further supplementation with *p*-aminobenzoic acid, nicotinic acid, and additional thiamin increases slightly the response of the Gebrüder Mayer strain to  $\beta$ -alanine. Riboflavin and folic acid have no effect. All of these B vitamins, however, are included at optimal concentrations in the medium of table 1 in the event that any of the yeasts studied require them for utilization of  $\beta$ -alanine or are stimulated by them. Choline, which inhibits the utilization of  $\beta$ -alanine by the Gebrüder Mayer strain in concentrations of 10  $\mu$ g per tube, is omitted.

Fifty  $\mu$ g adenine per tube increases the response of the Gebrüder Mayer yeast to large amounts of  $\beta$ -alanine, whereas equal quantities of xanthine have no effect. Both guanine and uracil inhibit at low concentrations of  $\beta$ -alanine. With the medium of Snell *et al.* (1940) and with the fully supplemented medium of table 1, maximum growth is observed at pH 4.5 to 5.0. Acetate ion inhibits growth in concentrations of M/100 or higher.

Gebrüder Mayer yeast cannot utilize the  $\beta$ -alanine or *l*-carnosine<sup>5</sup>; 200  $\mu$ g of *l*-carnosine support less growth than 0.1  $\mu$ g  $\beta$ -alanine. This is in contrast to the diphtheria bacillus (Mueller and Cohen, 1937) and to *Saccharomyces cerevisiae*, Fleischmann's strain 139 (Schenck and du Vigneaud, 1944), for which carnosine has about 25 per cent of the activity of  $\beta$ -alanine, on a molecular basis.

On the medium of table 1, smooth reproducible curves are obtained for the growth of Gebrüder Mayer yeast in the presence of  $\beta$ -alanine. However, many nitrogenous compounds inhibit the growth in the presence of small amounts of  $\beta$ -alanine. Various amino acids, casein digests, alkali-treated peptone, norit-treated beef, liver, and yeast extracts have been tested. For the sake of brevity only some of these results are shown in table 3. The medium of table 1 is used for these experiments.

At a level of 2 mg per tube, all preparations severely repress growth with small amounts of  $\beta$ -alanine. Cystine and aspartic acid are least inhibitory. In the presence of pantothenic acid or of 5  $\mu$ g  $\beta$ -alanine, however, there is no inhibition and in some cases stimulation is observed. The amount of stimulation of each substance is roughly the same in the presence of excess  $\beta$ -alanine or pantothenic acid. The following do not stimulate growth in the presence of excess  $\beta$ -alanine or pantothenic acid: *dl*-alanine, *l*-tryptophane, *dl*-valine, *dl*-methionine, and *dl*-leucine. Slight increases are obtained with *l*-histidine,

<sup>5</sup> Dr. Vincent du Vigneaud, Cornell University Medical School, kindly furnished the *l*-carnosine used in these studies.

*dl*-phenylalanine, *l*-cystine, *l*-tyrosine, and *dl*-threonine. The largest increments in growth are found after addition of vitamin-free hydrolyzed casein, norit-treated beef, liver, and yeast extracts, alkali-treated peptone, *l*-glutamic acid, *l*-lysine, *d*-arginine, asparagine, and glutamin. Higher levels of all these substances (5 or 10 mg per tube) inhibit the utilization of larger amounts of  $\beta$ -alanine. With 10 mg of asparagine per tube, the growth with 5  $\mu$ g  $\beta$ -alanine is less than that obtained with 0.1  $\mu$ g  $\beta$ -alanine on the asparagine-free medium.

Acid or alkali hydrolyzates of the norit-treated extracts are as inhibitory to  $\beta$ -alanine as are the original norit-treated extracts. Other unsuccessful attempts to offset the inhibition of the utilization of  $\beta$ -alanine by all of the above substances include the addition of small amounts of pantothenic acid (0.005 to

TABLE 3

*Effects of various substances upon the growth\* of Gebrüder Mayer yeast with  $\beta$ -alanine and calcium pantothenate*

CONTENTS PER TUBE		HY- DROLYZED CASEIN†	AS- PARA- GINE	ALKALI PEPTONE	<i>l</i> -CYSTINE	<i>l</i> -AS- PARTIC ACID	<i>l</i> -GLUTA- MIC ACID	<i>l</i> -TYRO- SINE	<i>dl</i> -LEU- CINE
$\mu$ g									
$\beta$ -Alanine									
0.0	0.025	0.012	0.016	0.015	0.027	0.025	0.025	0.017	0.020
0.1	0.280	0.018	0.018	0.015	0.130	0.050	0.040	0.020	0.020
0.2	0.520	0.017	0.020	0.015	0.550	0.270	0.060	0.025	0.035
5.0	0.570	0.820	0.700	0.730	0.650	0.620	0.720	0.590	0.555
Calcium panto- thenate									
0.1	0.260	0.320	0.300	0.280	0.240	0.270	0.445	0.245	0.250
0.2	0.490	0.710	0.560	0.565	0.500	0.530	0.650	0.480	0.470
2.0	0.580	0.840	0.700	0.760	0.630	0.640	0.740	0.600	0.560

\* Growth is expressed turbidimetrically in terms of optical density, which is equal to log 100 minus log per cent transmission ( $2 - \log G$ ).

† All of these substances were present at a level of 2 mg per tube.

0.01  $\mu$ g per tube), increasing the size of the inoculum up to fivefold, and using inocula from 16- and 24-hour liquid cultures.

While investigating the possibility of using yeast to measure  $\beta$ -alanine, an effective method for the quantitative separation of pantothenic acid from  $\beta$ -alanine was devised. Solutions containing 20  $\mu$ g pantothenic acid and 100  $\mu$ g  $\beta$ -alanine (separately or together) per 20 ml at pH 2.0 are treated with 300 mg of norit charcoal. The pantothenic acid is adsorbed, 98 to 100 per cent, whereas none of the  $\beta$ -alanine is removed. Norit is more effective than Darco G-60 for this separation, and can be successfully used with solutions from pH 3.5 to pH 1.

#### DISCUSSION

Yeasts presumably utilize  $\beta$ -alanine by converting it to pantothenic acid (Weinstock *et al.*, 1939). The lack of any stimulatory effect by lactone on the

growth with  $\beta$ -alanine may be explained by the ability of the yeasts to synthesize lactone faster than they can convert  $\beta$ -alanine to pantothenic acid. In this respect yeasts differ from *Acetobacter suboxydans*, which can utilize the lactone part of the pantothenic acid molecule for growth (Underkofler, Bantz, and Peterson, 1943), but which grows faster with lactone if  $\beta$ -alanine is also supplied<sup>6</sup>. However, the absence of inhibition of yeast growth with  $\beta$ -alanine by either taurine or pantoyltaurine (with one exception) is not clear in the light of the repression of pantothenic acid by pantoyltaurine. The inhibition of pantothenic acid is a competitive one (Snell, 1941), perhaps at the surface of a protein molecule, and it is possible that in the utilization of  $\beta$ -alanine by yeast, either  $\beta$ -alanine or lactone is attached to the protein molecule in question before being converted to pantothenic acid. However, the suggestion (Snell, 1941) that pantoyltaurine be used to inhibit pantothenic acid, and thereby measure only  $\beta$ -alanine, appears implausible, unless large amounts of pantoyltaurine are added, since the growth with  $\beta$ -alanine enables the yeast to utilize some of the pantothenic acid.

The addition of yeast extract increases the growth of all yeasts above that obtained with excess pantothenic acid or  $\beta$ -alanine. The addition of some amino acids, hydrolyzed casein, alkali-treated peptone, or norit-treated beef, liver, and yeast extracts also increases the growth above that observed with pure pantothenic acid, but these increases are not so great as those with whole yeast extract. This may be due to the presence of unknown stimulatory substances or of preformed vitamin conjugates in the yeast which make more rapid growth possible.

The inhibitory effects of amino acids, etc., on the utilization of small amounts of  $\beta$ -alanine limit the use of any of these yeasts, such as Gebrüder Mayer, to the measurement of  $\beta$ -alanine in solutions comparatively free of breakdown products of protein, such as commercial vitamin preparations. For this purpose, the pantothenic acid may be removed with norit. Pollack (1943) has found no  $\beta$ -alanine in digests of pure proteins, using Gebrüder Mayer yeast. This result may be due to inhibitory effects like those shown here for amino acids and casein hydrolyzate.

Recently Schenck and du Vigneaud (1944) presented a method for measuring the total  $\beta$ -alanine of tissues, using *S. cerevisiae*, Fleischmann's strain 139. However, the decreasing sensitivity of yeasts to  $\beta$ -alanine when increasing amounts of protein digests are added, as shown in the present paper, suggests that recoveries of added  $\beta$ -alanine and proportional growth at different levels of assay (which are not given by Schenck and du Vigneaud) would be erratic. The same considerations limit the method proposed by Cheldelin and Mitchell (1943).

#### SUMMARY

A comparison of the 18-hour growth of 16 strains of *Saccharomyces cerevisiae* and one of *Saccharomyces carlsbergensis* on a synthetic medium with various

<sup>6</sup> Sarett and Cheldelin, unpublished observations.

levels of  $\beta$ -alanine and pantothenic acid is presented. The yeasts differ in their sensitivity to  $\beta$ -alanine and pantothenic acid. Strain 2190 cannot utilize  $\beta$ -alanine at all, whereas strain Gebrüder Mayer is most responsive to very small amounts of  $\beta$ -alanine. Some of the yeasts do not attain so much growth with excess  $\beta$ -alanine as with excess pantothenic acid. The addition of yeast extract increases the growth above that obtained with excess pantothenic acid with all yeasts.

The response to  $\beta$ -alanine is not affected by the presence of an excess of the lactone of the pantothenic acid molecule, and only one yeast is inhibited in its growth with  $\beta$ -alanine by large amounts of taurine or pantoyltaurine. Pantoyltaurine inhibits the utilization of pantothenic acid for growth by all the yeasts. The utilization of small amounts of  $\beta$ -alanine is inhibited by asparagine.

For the Gebrüder Mayer yeast, the growth in the presence of small amounts of  $\beta$ -alanine is inhibited by amino acids, hydrolyzed casein, and norit-treated beef, liver, and yeast extracts. The response to larger amounts of  $\beta$ -alanine and all levels of pantothenic acid is either not affected or is stimulated by these same substances. The limitations of the use of yeasts for measurement of  $\beta$ -alanine are discussed.

A method for the quantitative separation of pantothenic acid and  $\beta$ -alanine is presented.

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# THE PANTOTHENIC ACID REQUIREMENTS OF LACTIC ACID BACTERIA<sup>1</sup>

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Advances in vitamin and amino acid research are being speeded by the successful use of lactic acid bacteria as assay organisms. Their fastidious growth requirements and the ease of measuring the extent of their growth or acid production render them ideally suited for studies in this field. Interest in these organisms has centered about the nutritional requirements of *Lactobacillus casei* and *Lactobacillus arabinosus*, which have been used extensively for the measurement of several B vitamins and amino acids.

Research on the growth requirements of other species of lactic acid bacteria has been extended for other vitamin problems. Special studies have been made of *Streptococcus lactis* R (Mitchell, Snell, and Williams, 1941), *Leuconostoc mcsenteroides* (Gaines and Stahly, 1943), and *Lactobacillus fermentum*<sup>2</sup> 36 (Sarett and Cheldelin, 1944).

The present paper is a survey of the pantothenic acid nutrition of 33 species and strains of these bacteria. Growth comparisons are compiled using different media, and evaluations are made of the various organisms for possible use in the assay of pantothenic acid.

## EXPERIMENTAL

*Culture media.* Four culture media have been used in this study. Their components are listed in table 1. Medium A is a synthetic type to which acid-hydrolyzed vitamin-free casein is added. Media B, C, and D contain in addition pantothenic-acid-free extracts of peptone or yeast. All of the known B vitamins and several other growth-stimulating substances are present in all media except C. The latter is essentially that of Pennington, Snell, and Williams (1940) with the exception that the glucose and sodium acetate concentrations of the original medium have been increased to the amounts recommended by Stokes and Martin (1943).

*Cultures.* Cultures were generously supplied to us from various laboratories and included members of the genera *Lactobacillus*, *Streptococcus*, and *Leuconostoc*,

<sup>1</sup> Presented at the second meeting of the Oregon Academy of Science, Portland, 1944.

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<sup>2</sup> The correct name of this organism has been suggested by Dr. J. M. Sherman to be *Lactobacillus fermenti*.



as follows:<sup>3</sup> *Lactobacillus casei*, *L. acidophilus* (2 strains), *L. plantarum*, *L. arabinosus* (2 strains), *L. pentosus* (2 strains), *L. delbrueckii* (2 strains), *L. brevis* (2 strains), *L. buchneri* (2 strains), *L. pentoaceticus*, *L. lycopersici*, *L. mannilopoeus*, *L. fermenti* (2 strains), *L. gayoni*, *L. brassicae*, *Streptococcus lactis* (4 strains), *S. liquefaciens*, *S. durans*, *S. zymogenes*, *Leuconostoc mesenteroides*, and *Leuconostoc dextranicus*.

TABLE 1  
*Ingredients of growth media*

	A	B	C	D
Acid-hydrolyzed technical casein,* g.....			4	
Acid-hydrolyzed vitamin-free casein,† g.....	10	10		10
Alkali-treated peptone,* g.....			10	10
Glucose, g.....	40	40	40	40
Sodium acetate, anhydrous, g.....	36	36	24	24
Alkali-treated Difco yeast extract,* g.....		2	2	2
Cystine hydrochloride, mg.....	200	200	200	200
Tryptophane, mg.....	200	200		200
Asparagine, mg.....	1	1		1
Adenine sulfate, mg.....	20	20		20
Guanine hydrochloride, mg.....	20	20		20
Xanthine, mg.....	20	20		20
Uracil, mg.....	20	20		20
Thiamin hydrochloride, µg.....	200	200		200
Riboflavin, µg.....	400	400	200	400
Nicotinic acid, µg.....	200	200		200
Pyridoxine hydrochloride, µg.....	200	200		200
Biotin, (free acid), µg.....	0.8	0.8		0.8
Inositol, mg.....	5	5		5
p-Aminobenzoic acid, µg.....	200	200		200
Choline chloride, mg.....	2	2		2
Folic acid,‡ µg.....	5	5		15
Inorganic salts,§ A and B, ml.....	10	10	10	10
Distilled water to 1 liter; pH 6.6-6.8.				

\* Pennington, Snell, and Williams (1940).

† Snell and Wright (1941).

‡ A folic acid concentrate was kindly furnished by Dr. R. J. Williams of the University of Texas, and is used here in terms of 40,000 potency.

§ Snell and Strong (1939).

*Technique of testing responses.* Experiments are carried out in 20 x 150 mm, lipless, pyrex test tubes to which the substances to be tested are added, diluted to 5 ml, and 5 ml of the appropriate medium added. The organisms for inocula are grown similarly in medium C to which 0.2 µg of pantothenic acid, 5 mg of

<sup>3</sup> We are indebted to the following persons for several of the bacterial cultures used: W. B. Bollen and J. E. Simmons, Oregon State College; R. J. Williams and E. E. Snell, University of Texas; E. McCoy and W. H. Peterson, University of Wisconsin; L. A. Burkey, U. S. Dept. of Agriculture; C. S. Pederson, N. Y. Agricultural Expt. Station; B. W. Hammer, Iowa State College.

liver extract<sup>4</sup> and 5 mg of Difco yeast extract have been added. These are centrifuged after 16 to 24 hours of growth, resuspended, and washed twice with sterile 0.9 per cent NaCl solution. One drop of each final suspension is added to the appropriate tubes for testing.

Cultures of *L. brevis* L35, *L. gayoni* F20, *L. brassicae*, *Leuconostoc dextranicus*, *Leuconostoc mesenteroides*, *S. lactis* R8043, *S. lactis* RG1A, *S. lactis* 374, *S. liquefaciens* (2 strains), and *S. zymogenes* are grown at 30 C. All others are grown at 37 C. Turbidity readings are observed in a Pfaltz and Bauer fluorophotometer equipped with a special holder for the tubes which have been used throughout the study. With uniform tubes it is possible to make several turbidity measurements during the course of each experiment.

TABLE 2

*Response of lactic acid bacteria to pantothenic acid and its constituent moieties*

ORGANISM	Medium	TURBIDITY* AFTER 24 HOURS				ML 0.1 N ACID PRODUCED IN 72 HOURS							
		$\mu$ g Pantothenic acid per 10 ml culture†				$\mu$ g Pantothenic acid per 10 ml culture†				Medium	5 $\mu$ g $\beta$ -alanine	5 $\mu$ g lactone	5 $\mu$ g $\beta$ -al. + 5 $\mu$ g lact.
		0	0.2	1	1 + YL‡	0	0.2	1	1 + YL‡				
<i>L. delbrueckii</i> 72 (Group I)§	A	0.01	0.53	0.80	1.00	0.1	6.0	14.0	16.4	C	1.3	1.5	1.4
	B	0.01	0.61	0.92	1.00	0.2	6.1	14.9	16.4	C	1.3	1.4	1.4
<i>L. arabinosus</i> 17-5 (Group II)	A	0.04	0.32	0.56	0.96					C	1.8	1.8	1.8
	B	0.06	0.41	0.75	1.00	1.0	12.5	14.5	18.5				
	C	0.07	0.64	1.35	1.50	1.8	15.4	18.6	19.9				
<i>L. casei</i> (Group III)	A	0.02	0.05	0.06	0.64	0.1	3.3	6.6	15.6	C	2.0	2.0	2.0
	B	0.02	0.06	0.08	0.89	1.1	5.4	9.8	18.5				
	C	0.07	0.35	0.55	0.73	1.9	13.8	17.6	20.0				
	D	0.07	0.76	1.22	1.25	1.9	13.6	19.9	19.1				

\* Turbidity is given in terms of optical density.

† Pantothenic acid quantities are given in terms of calcium pantothenate.

‡ YL = 5 mg yeast extract plus 5 mg liver extract.

§ Group numbers are described in the text, along with the group classification of the other 30 cultures studied.

Turbidity values after 24 hours' growth are reported in terms of optical density (log 100 minus log galvanometer reading). Production of lactic acid after 3 days' growth has been determined in all experiments by titration with 0.1 N alkali. Turbidity readings after 3 days are not shown as they correlate generally with the amount of acid produced. Since it has been impossible to perform the experiments on all of the organisms at one time, *L. casei* is included in every set for purposes of comparison.

*Results.* The response to pantothenic acid by the different organisms in the four growth media is summarized in table 2. All of the organisms tested are

<sup>4</sup> We are indebted to Dr. T. H. Jukes of the Lederle Laboratories, Pearl River, New York, for the liver extract used.

seen to require pantothenic acid for growth, but they are unable to utilize the  $\beta$ -alanine and lactone<sup>6</sup> moieties. The results confirm and extend earlier observations (Snell, 1941; Snell, Strong, and Peterson, 1939; and several others).

Tubes containing added yeast and liver extracts are included with each series for comparison. It is assumed that growth in the presence of these extracts is the maximum for each organism, and a medium is adjudged to satisfy the nutrient requirements of the organism if the response to added pantothenic acid is essentially equal to that obtained with these added extracts. On this basis the organisms have been classified into three groups, which are described below.

None of the 33 cultures are able to reach maximum growth in the absence of peptone, yeast, and liver extracts (medium A). Those organisms which attain maximum growth with pantothenic acid added to a medium containing alkali-treated yeast (medium B) are classified in group I. The four organisms in this group are *L. delbrueckii* 72, *L. delbrueckii* 3, *L. fermenti* 76, and *S. durans*. These organisms attain as much growth and produce as much acid (16 to 18 ml) on medium B with 1  $\mu$ g of pantothenic acid as with added yeast and liver extracts. For *S. durans*, maximum acid production is only 9 to 10 ml. Of these four organisms, *L. delbrueckii* 72 shows the best relative response on medium A.

In group II are organisms which grow optimally in the presence of alkali-treated yeast and peptone with no added B vitamins except riboflavin (medium C). Most of the organisms tested may be placed in this group. Some of these grow extremely rapidly. The cultures become very turbid in 10 to 14 hours and produce 18 to 20 ml of acid in three days. These are *L. arabinosus* 17-5, *L. arabinosus* 8014, *L. pentosus* 124-2, *L. pentosus* 8041, *L. plantarum* 8292, *L. pentoaceticus* 367, and *L. buchneri* K14. Other organisms in this classification which produce less acid (up to 10 ml) are *L. brevis* 118-8, *L. brevis* L35, *L. lycopersici* 4005, *S. lactis* RG1A, *S. lactis* 374, *S. lactis* R8043, *S. zymogenes*, *Leuconostoc dextranicus*, *Leuconostoc mesenteroides* Pd60, *S. liquefaciens* (Oregon State College), and *L. gayoni* F20.

Organisms which grow optimally only if the medium is supplied with some or all of the known B vitamins in addition to alkali-treated yeast and peptone extracts are included in group III. In this group are *L. acidophilus* 832, *L. acidophilus* (Oregon State College), *L. brevis*, *L. casci*, *L. buchneri*, *L. fermenti* 36, *L. brassicae*, *S. liquefaciens* (Iowa State College), and *S. lactis* 125. The first 4 of these organisms produce approximately 20 ml of acid per culture; the others produce 8 to 10 ml.

In addition, one organism, *L. mannitopoeus*, has been observed to reach maximum growth on medium D only when *untreated* yeast and liver extracts are included. Acid production in the absence of these extracts is only about 60 per cent of the maximum obtainable.

#### DISCUSSION

The type of medium represented by C appears well suited for routine work, since it contains relatively few ingredients and is easy to prepare. Moreover,

<sup>6</sup> Throughout this paper, the lactone moiety of the pantothenic acid molecule refers to (-)- $\alpha$ -hydroxy- $\beta$ , $\beta$ -dimethyl- $\gamma$ -butyrolactone. This compound was kindly supplied by Dr. J. C. Keresztesy, Merck & Co., Rahway, New Jersey.

the pantothenic-acid-free extracts of peptone and yeast supply the organisms with a number of growth-promoting substances, some of which are as yet uncharacterized.

The first seven cultures listed in group II possess desirable features for use as assay organisms for pantothenic acid. They grow and produce acid more rapidly than does *L. casei*, a group III organism, which is generally used for assay purposes. Further experiments with these group II organisms have shown *L. arabinosus* 17-5 to be useful for pantothenic acid assay (Hoag, Sarett, and Cheldelin, 1944).

For the study of vitamins other than pantothenic acid, the special growth requirements of the Group III organisms may be useful. Thus, *L. casei* is stimulated by folic acid. Thiamin is necessary for growth of *L. fermenti* 36, and its use for assay purposes is described elsewhere (Sarett and Cheldelin, 1944). The remaining organisms are not stimulated by folic acid or thiamin.

#### SUMMARY

Pantothenic acid has been found to be a growth determinant for 33 strains of lactic acid bacteria. The  $\beta$ -alanine and lactone moieties of pantothenic acid are not utilized by these organisms.

Several organisms which produce large amounts of lactic acid on a relatively simple medium appear well suited for the assay of pantothenic acid. Growth and acid production have been compared using 4 media. Optimum response of most species can be obtained in the presence of added pantothenic acid by supplementing a simple growth medium with extracts of peptone and yeast which are free of pantothenic acid.

The addition of other B vitamins to this medium is utilized in studying the other requirements of some species.

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# EFFECTS OF ENZYME PREPARATIONS UPON PENICILLIN

## I. A METHOD FOR TESTING PENICILLIN FOR STERILITY

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The necessity of adapting or modifying standard or official sterility test methods for products known to possess bacteriostatic properties has resulted in the development of improved media for testing biological preparations. The approved culture medium now used for the sterility test is "fluid thioglycollate medium" as prepared by the Brewer or Linden methods which are described in the National Institute of Health memorandum dated December 30, 1941. This medium supports both aerobic and anaerobic growth and particularly neutralizes the mercurial preservatives added to some pharmaceuticals.

Following the observation that *p*-aminobenzoic acid and related compounds would neutralize completely the antibacterial effects of sulfonamides (Woods, 1940; Woods and Fildes, 1940; Keltch *et al.*, 1941), sterility media containing appropriate amounts of *p*-aminobenzoic acid have been recommended for testing sulfonamides for the presence of bacterial contamination (Brewer, 1943; McLintock and Goodale, 1943). With the introduction of penicillin, which is considerably more effective than the sulfonamides in the treatment of systemic and wound infections caused by many gram-positive and some gram-negative bacteria (Abraham *et al.*, 1941; Bordley *et al.*, 1942; Florey and Florey, 1943; Herrell, 1943; Herrell *et al.*, 1943; Rammelkamp and Keefer, 1943), the necessity of providing a suitable method for neutralizing this highly active antibacterial agent during sterility testing became apparent. An acceptable procedure for this purpose should completely inactivate penicillin without destroying bacteria or spores present as possible contaminants. It should be rapid and simple, and permit maintenance of sterile conditions; the addition of inactivated penicillin should result in minimal changes in the usual sterility test media.

Among the various agents tested which appeared to meet the stated requirements was an enzyme system, taka-diastase, which was found to inactivate penicillin completely in 2 hours or less when incubated with it in a water bath at 40 C and tested by the Oxford cup-plate method (Abraham *et al.*, 1941). The enzyme was furthermore found to have little, if any, antibacterial action when used alone in the same test procedure.

Following these preliminary observations, studies were extended to include various proteolytic and carbohydrate-splitting enzymes, among which were several of plant and microbial origin. The commonly known proteolytic enzyme systems studied were pepsin, trypsin, crepsin, pancreatin, and papain. To this list were added ficin, a protein-splitting enzyme obtained from the latex of certain species of the fig tree; hurain, a new ferment which was isolated from

the sap of the tree, *Hura crepitans*; and a proprietary product "polidase," a cultured vegetable enzyme preparation containing proteolytic as well as amylolytic systems. The carbohydrate-splitting enzymes tested included invertase, malt diastase, and emulsin. Several amylase preparations, namely, fungal amylase, taka-diastase, clarase, and mylase "P," all derived from the mold, *Aspergillus oryzae*, were added to this group. A bacterial amylase preparation which was prepared from *Bacillus mesentericus* completed the series of carbohydrate ferments. Catalase and egg-white lysozyme, two enzyme systems which do not belong to either of the two groups given above, were also studied.<sup>1</sup>

1. *Test Methods.* Stock 1 per cent solutions or suspensions of the enzyme preparations were prepared by adding the dry powders to buffer solutions of pH 4.0, 6.0, and 8.0. To 1 ml of the enzyme solution, in a 15 x 200 mm test tube, was added 1 ml of an aqueous solution of sodium penicillin containing approximately 40 to 48 Oxford units. The test mixtures, along with suitable controls, namely, buffer solutions alone as well as penicillin and buffers without enzyme, were placed in a water bath at 40 C. Following 2 hours' incubation, and again at the end of 18 hours, a small quantity of test solution was removed from the tubes and tested by the Oxford cup-plate method for penicillin activity. The results of this study are presented in table 1. Mention should be made of the fact that cysteine hydrochloride (0.5%) was added to the papain mixture in order to activate the enzyme. Furthermore, since aqueous solutions of the amino acid are distinctly acid, this factor was taken into consideration when adjusting the papain cysteine mixture to the pH values used in the study.

From the data given in table 1, and particularly the results at the end of the 2-hour incubation period, the following points may be noted. The papain cysteine mixture appeared to inactivate the antibacterial effects of penicillin completely in the presence of the buffers at pH 6.0 and 8.0 but not at 4.0. Bacterial amylase and taka-diastase were equally effective in this same action in 2 hours or less at pH 6.0 and 8.0 but neutralized penicillin activity completely at pH 4.0 only after more than 2 hours' action. The most active preparation studied was clarase, which inhibited the antibacterial effects of penicillin completely under the experimental conditions given at all the pH ranges tested. While the remaining enzymes failed to bring about complete neutralization of the agent following incubation for 2 hours at 40 C, some were noted to be effective in this action when tested at the end of 18 hours.

The protein-splitting enzymes, pepsin, trypsin, erepsin, pancreatin, ficin, and hurain, gave little, if any, evidence of antipenicillin activity at the end of the 2-hour test period. The apparent neutralizing effects of practically all

<sup>1</sup> The several enzymes mentioned above were secured from the following sources: ficin from Merck and Company, Inc., Rahway, N. J.; hurain from Dr. W. G. Jaffe, Caracas-Los Rosales, Venezuela; polidase from the Schwartz Laboratories, Inc., New York, N. Y.; invertase, fungal amylase, mylase "p," and the preparation from *B. mesentericus* from Wallerstein Laboratories, Inc., New York, N. Y.; emulsin from the National Bureau of Standards, U. S. Department of Commerce, Washington, D. C.; clarase from the Takamine Laboratories, Inc., Clifton, N. J.; and lysozyme from Stein Hall & Company, New York, N. Y.

enzymes upon penicillin in buffer at pH 4.0, when tested at the 18-hour incubation period, may be attributed to the effects of the acidity of the medium upon the agent rather than an enzyme action. Although it is not indicated in the table, control enzyme solutions or mixtures alone, in the absence of penicillin, failed in all instances to show any evidence of inhibition of the development of the test organism, *Staphylococcus aureus*, in the agar cup plates.

Several of the enzyme preparations showing definite antipenicillin activity were studied further.

TABLE 1  
*The effects of several enzyme systems upon penicillin*

ENZYME SYSTEM	pH BUFFER—MEDICATION TIME—MM INHIBITION					
	pH 4.0		pH 6.0		pH 8.0	
	2 hr	18 hr	2 hr	18 hr	2 hr	18 hr
1. Pepsin . . . . .	29	0	33	34	33	33
2. Trypsin . . . . .	32	0	32	31	33	32
3. Erepsin . . . . .	31	0	33	33	32	31
4. Pancreatin . . . . .	31	0	33	32	32	33
5. Papain cysteine . . . . .	31	11	0	0	0	0
6. Ficin . . . . .	32	12	32	33	32	33
7. Ilurain . . . . .	27	0	33	27	34	29
8. Polidase . . . . .	33	11	30	34	33	32
9. Invertase . . . . .	32	0	32	33	33	32
10. Malt diastase . . . . .	29	0	32	29	32	0
11. Mylase "P" . . . . .	33	0	33	26	30	31
12. Fungal amylase . . . . .	32	12	32	26	33	31
13. Bacterial amylase . . . . .	28	0	0	0	0	0
14. Clarase . . . . .	0	0	0	0	0	0
15. Taka-diaastase . . . . .	23	0	0	0	0	0
16. Emulsin . . . . .	31	0	32	28	32	28
17. Catalase . . . . .	33	11	32	34	33	32
18. Lysozyme . . . . .	32	0	31	14	27	0
Penicillin buffer (control)	29	0	31	33	33	34

Figures represent mm inhibition exhibited by penicillin against *Staphylococcus aureus* by the Oxford cup-plate test (outside diameter of cups measure 8 to 9 mm); 0 = penicillin has been inactivated, hence no inhibition demonstrable.

II. *Papain-Cysteine*. Since it was felt that the cysteine hydrochloride in the papain cysteine mixture might play an important part in neutralizing the antibacterial effects of penicillin because of the reducing action of the amino acid upon the latter substance, papain alone and cysteine hydrochloride alone were tested against the antibacterial agent. As additional controls, cystine alone and a mixture of cystine and papain were included in the test. The results of these studies are presented in table 2. From the data it seems evident that the reducing action of cysteine is responsible for inactivation of penicillin in the papain cysteine penicillin mixture (table 1). The papain cystine combination failed completely to neutralize the antibacterial effects of the antibiotic substance.



The inactivating action of cysteine upon penicillin suggested the possible use of the amino acid alone in testing the agent for sterility. In using the reducing substance, however, certain factors had to be taken under consideration. Solutions of cysteine hydrochloride are distinctly acid in reaction; therefore, as mentioned previously, adjustment of the pH of solutions of the amino acid must be made before they are used in the sterility test. Our studies, furthermore, indicated that the equivalent of 10 to 20 mg of cysteine hydrochloride are necessary to inactivate 1,000 to 2,000 units of penicillin. In order to obtain a maximum neutralizing effect of the reducing agent on the antibacterial substance, solutions or mixtures of the two preparations should be incubated 15 to 20 minutes prior to addition to the sterility medium.

Although not fully meeting the necessary requirements for a practical procedure to be used to inactivate penicillin in the sterility test, the use of neutral solutions of cysteine hydrochloride for this purpose appears possible.

TABLE 2  
*Effects of papain in the presence of cysteine and cystine upon penicillin activity*

ENZYME 1% AMINO ACID 0.5%	pH BUFFER—MEDICATION TIME—MM INHIBITION					
	pH 4.0		pH 6.0		pH 8.0	
	2 hr	18 hr	2 hr	18 hr	2 hr	18 hr
Papain + cysteine.....	33*	12	0	0	0	0
Cysteine (control).....	33	14	0	0	0	0
Papain + cystine.....	34	12	34	33	34	32
Cystine (control).....	34	0	32	30	34	29
Papain + penicillin (control).....	33	11	34	32	33	30
Papain—no penicillin (control).....	0	0	0	0	0	0
Penicillin only.....	34	11	35	34	34	33

\* See legend under table 1.

III. *Taka-Diastase and Clarase.* Note should be made of the fact that the clarase preparation used in all preliminary studies and in most of the investigation presented in this communication was the product known as standardized clarase (lot no. 962). A concentrated clarase (lot no. 1455) was made available for our studies by the Takamine Laboratories. The difference between the two types of clarase is that the concentrated form is enzyme only, whereas the standardized clarase contains milk sugar as a diluent, which is added to standardize the preparation, in which form it is recommended for assay tests. A comparison of the relative degrees of penicillin inactivation exhibited by these two preparations was made from time to time; however, unless indicated otherwise, the data given are based upon the use of standardized clarase solutions.

The extent to which the two clarase preparations, as well as taka-dia-*stase*, may be diluted and still show evidence of neutralizing the antibacterial effects of penicillin was determined by the following method. Dilutions of the enzymes

were prepared from 1 per cent stock solutions to give concentrations of 1:250, up to and including 1:100,000, in phosphate buffer solution of pH 7.0. To 1 ml of sodium penicillin solution containing 40 to 48 Oxford units was added 1 ml of one of the enzyme dilutions. Following incubation at 40 C for 2 hours and 18 hours, samples were tested for penicillin activity by the Oxford cup-plate method.

The results of these tests, and particularly those at the end of 2 hours' incubation, revealed the following: concentrated clarase neutralized the antibacterial effects of penicillin completely in a dilution of 1:10,000; standardized clarase gave a similar effect in dilutions up to and including 1:4,000, whereas taka-diastase was effective at the 2-hour period only when used in the undiluted form of 1:100. Tests made at the end of 18 hours indicated that the 1:25,000 dilutions of both concentrated and standardized clarase gave comparable degrees of penicillin inactivation under the experimental conditions mentioned. A similar effect was noted with taka-diastase in the 1:250 dilution. On the basis of these findings, it is evident that the substance or substances responsible for antipenicillin activity are far more active or are present in much greater quantities in clarase than in taka-diastase.

IV. *Activities of Taka-Diastase and Clarase upon Penicillin in the Presence of a Nutrient Fluid Medium.* Since it was found that relatively high dilutions of clarase (standardized) would completely inactivate the antibacterial effects of penicillin, as demonstrated by the Oxford cup-plate procedure, it appeared worth while to study the effects of the enzyme preparation upon the agent in the presence of broth. Taka-diastase was included in this study for comparative purposes.

One per cent stock solutions of the enzymes were prepared in buffer solution, pH 7.0. The solutions were sterilized by Berkefeld filtration. Dilutions were made from the stock, again in buffer solution, to give concentrations of 1:250, 1:500, 1:1,000, 1:1,500, and 1:2,000. A solution of sodium penicillin was prepared to contain approximately 40 to 48 Oxford units per ml. This material was sterilized by boiling for 3 minutes and cooled rapidly to room temperature. The treatment had no apparent effect upon penicillin potency.

To 2.5-ml. quantities of penicillin, in sterile test tubes, was added an equal volume of one of the enzyme dilutions. Transfers of 1 ml of the test mixtures were made immediately to 9 ml of Brewer's fluid thioglycollate medium. Similarly, control buffer solutions without penicillin, clarase and taka-diastase solutions alone, and penicillin in buffer solution without enzyme were added to individual tubes of the test medium.

Each of a series of test solutions was inoculated with *Clostridium tetani*, *Clostridium septique*, and *Staphylococcus aureus* 209. The inoculum in each instance consisted of a 4-mm loopful of a 3-day Brewer's fluid thioglycollate medium culture of test organism. The tubes were incubated at 37 C and observed daily for a period of 7 days for the presence of visible growth.

The data which are presented in table 3 clearly indicate the superiority of

clarase over taka-diastase in neutralizing the antibacterial effect of penicillin. The highest dilution of clarase tested, 1:40,000, permitted growth of the test organism in the presence of the antibiotic substance. A similar antipenicillin effect was obtained with taka-diastase in the 1:2,000, 1:5,000, and 1:10,000 dilutions against *C. tetani*, *S. aureus*, and *C. septicus*, respectively. The tubes containing cultures and penicillin without enzymes were devoid of visible growth throughout the 7-day test period. The control tubes containing enzymes and organisms but no penicillin showed evidence of growth of the test bacteria at the end of 24 to 48 hours of incubation.

The extent to which clarase could be diluted and still be effective in neutralizing the antibacterial effects of penicillin in the presence of a fluid nutrient medium appeared to merit consideration. Concentrated clarase and taka-diastase were included in the study for comparative purposes. *Staphylococcus*

TABLE 3  
*Effect of clarase and taka-diastase upon penicillin in presence of fluid medium*

FINAL ENZYME CONCENTRATION IN BROTH	ORGANISM—ENZYME—GROWTH AT 7 DAYS					
	<i>C. tetani</i>		<i>C. septicus</i>		<i>S. aureus</i>	
	TD	C	TD	C	TD	C
1:2,000 . . . . .	+	+	+	+	+	+
1:5,000 . . . . .	0	+	+	+	+	+
1:10,000 . . . . .	0	+	+	+	0	+
1:20,000 . . . . .	0	+	0	+	0	+
1:30,000 . . . . .	0	+	0	+	0	+
1:40,000 . . . . .	0	+	0	+	0	+
Enzyme only 1:2,000 . . . . .	+	+	+	+	+	+
Penicillin only . . . . .	0	0	0	0	0	0
Broth culture (control). . . . .	+	+	+	+	+	+

TD = taka-diastase, C = clarase (standardized); + = growth to extent noted in control broth tube; 0 = no visible growth, indicating antibacterial effect of penicillin.

*aureus* was used as the test organism. The details of the test method are as follows: 8 ml of Brewer's medium were distributed in a series of cotton-plugged test tubes. The medium was sterilized by autoclaving at 10 pounds for 10 minutes. Upon cooling, to each tube was added 1 ml of a sterile sodium penicillin solution and 1 ml of an appropriate dilution of sterile enzyme solution. After the contents of the tubes were mixed thoroughly by swirling, a 4-mm loopful of a 24-hour broth culture of the test organism was added. The inoculated tubes, with suitable controls, were placed at 37 C to incubate and observed for the presence of growth at the end of 1, 3, 5, and 7 days.

The data (table 4) show that the test organism in the presence of a mixture of penicillin and 1:100,000 concentrated clarase grew luxuriantly within a 24-hour incubation period. The same extent of growth was noted in the presence of penicillin containing 1:10,000 standardized clarase and the 1:2,000 dilution of taka-diastase. Following 3 to 5 days of incubation, all tubes containing

concentrated clarase and penicillin showed evidence of maximum growth of the test organism. Standardized clarase permitted maximum growth at the end of 5 days. Taka-diastrase was far inferior in its neutralizing effect upon penicillin activity, allowing a good growth of the test organism at the end of 7 days only in the tubes containing 1:2,000 to 1:20,000 dilutions of the enzyme system. These findings confirm the results obtained in the Oxford cup-plate dilution tests using these 3 enzyme preparations, wherein the degree of effectiveness of the compounds in inhibiting penicillin activity was in the order given in the present study.

TABLE 4

*Comparison of antipenicillin effects of dilute solutions of clarase and taka-diastrase in nutrient broth*

FINAL CONCENTRATION OF ENZYMES IN TEST MEDIUM CONTAINING PENICILLIN	OBSERVATION-DAYS, ENZYME PREPARATIONS, DEGREE OF GROWTH											
	1st			3rd			5th			7th		
	CC	SC	TD	CC	SC	TD	CC	SC	TD	CC	SC	TD
1:2,000 . . . . .	4	4	4	4	4	4	4	4	4	4	4	4
1:5,000 .. . . .	4	4	0	4	4	4	4	4	4	4	4	4
1:10,000 . . . .	4	4	0	4	4	0	4	4	2	4	4	4
1:20,000 . . . .	4	0	0	4	4	0	4	4	1	4	4	3
1:30,000 . . . .	4	0	0	4	4	0	4	4	0	4	4	0
1:40,000 . . . .	4	0	0	4	4	0	4	4	0	4	4	0
1:50,000 . . . .	4	0	0	4	4	0	4	4	0	4	4	0
1:60,000 . . . .	4	0	0	4	3	0	4	4	0	4	4	0
1:70,000 . . . .	4	0	0	4	2	0	4	4	0	4	4	0
1:80,000 . . . .	4	0	0	4	2	0	4	4	0	4	4	0
1:100,000 . . .	3	0	0	4	1	0	4	4	0	4	4	0
1:120,000 . . .	0	0	0	4	0	0	4	3	0	4	4	0
1:140,000 . . .	0	0	0	4	0	0	4	3	0	4	4	0
1:160,000 . . .	0	0	0	3	0	0	4	2	0	4	4	0
1:200,000 . . .	0	0	0	3	0	0	4	0	0	4	4	0
1:250,000 . . .	0	0	0	3	0	0	4	0	0	4	4	0
Control	4											

CC = concentrated clarase; SC = standardized clarase; TD = taka-diastrase; 1 to 4 = slight to maximum growth.

V. *Bacterial Amylase*. The results of the studies on the bacterial amylase preparation (table 1) have not been elaborated upon in this report inasmuch as its degree of antipenicillin action was comparable to that of taka-diastrase.

Since the original purpose of the present investigation was to find a suitable means of testing penicillin for sterility, the use of clarase for neutralizing the antibacterial effects of the agent appeared possible. This conjecture was based upon the data presented above and particularly on studies which more closely duplicated conditions encountered in actual sterility test procedures in the control laboratories.

VI. *Use of Clarase in Sterility Test for Penicillin*. Ampules of sodium penicillin powder received in the laboratory for sterility testing were inoculated with

dry cultures of *C. tetani*, *C. septicus*, *Bacillus subtilis*, *Eberthella typhi*, *Escherichia coli*, and *S. aureus*. Two ml of a sterile 1 per cent aqueous solution of clarase (buffered at pH 7.0) were added to the contaminated powders, which were then transferred immediately to several tubes of Brewer's fluid thioglycollate medium. The contents of the tubes were mixed thoroughly by swirling and placed at 37 C to incubate. Growth of the inoculated test organisms occurred in all tubes at the end of 48 hours' incubation. All tubes containing gram-positive organisms and penicillin without enzyme failed to show evidence of visible growth at the end of 7 days; however, many of the tubes containing gram-negative organisms and penicillin without enzyme showed some growth after several days. These results might have been expected on the basis of present knowledge of the relative ineffectiveness of penicillin on many gram-negative bacteria *in vitro*, particularly against organisms of the colon typhoid dysentery group.

In view of these findings, the following supplement to standard sterility test methods has been proposed (Lawrence, 1943). A stock solution of sterile 1 per cent clarase is divided into 2-ml amounts in sterile cotton-plugged test tubes or ampules (the sterile enzyme solution, when stored in a refrigerator, will retain its antipenicillin activity for a period of more than two months). The contents of an ampule of penicillin powder are dissolved or suspended in the 2-ml quantity<sup>2</sup> of clarase solution and transferred to tubes containing sterility medium. The test solutions are placed at 37 C and examined for visible bacterial contamination for 7 days. An additional 7 days may be allowed for detection of possible mold contamination.

In the absence of a suitable antipenicillin agent, such as the active clarase preparations used in this study, the antibiotic substance alone in thioglycollate medium will still be found to be sufficiently bacteriostatic at the end of 30 days' incubation at 37 C to inhibit completely the growth of a large inoculum of *Staphylococcus aureus*.

#### SUMMARY

In developing a suitable method for testing penicillin for sterility, several enzyme preparations appeared to meet the necessary requirements for inactivating the antibiotic substance.

With one exception the enzymes showing antipenicillin effects were proprietary products, taka-diastase and clarase, both of which are derived from the fungus, *Aspergillus oryzae*. A bacterial enzyme preparation from *Bacillus mesentericus* was also found to neutralize the antibacterial effects of penicillin.

Other diastatic enzyme systems, including several also derived from *Aspergillus oryzae* as well as a group of proteolytic enzymes, failed completely to show any evidence of an antipenicillin effect.

On the basis of the results obtained with a highly active, antipenicillin clarase preparation, a sterility test using solutions of the latter has been proposed.

<sup>2</sup> This quantity of standardized clarase (lot 932) solution was found to inactivate as much as 50 mg of penicillin sodium having a total potency of 10,000 or more Oxford units.

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# EFFECTS OF ENZYME PREPARATIONS UPON PENICILLIN

## II. AGENTS RESPONSIBLE FOR PENICILLIN INACTIVATION

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In a previous communication (Lawrence, 1945) were presented the relative effects of various enzyme systems or preparations upon the antibacterial action of penicillin. Of the several proteolytic and amylolytic ferments studied two products, taka-diastase and clarase, both derived from the fungus *Aspergillus oryzae*, were found to neutralize or inactivate completely the antibiotic substance. Bacterial amylase, a preparation obtained from *Bacillus mesentericus*, also proved to be antagonistic to the antibacterial properties of penicillin.

Other diastatic ferments, including several also derived from *Aspergillus oryzae*, failed completely to show any evidence of an antipenicillin effect. On the basis of these findings, it seemed worth while to extend the studies on the active preparations in an attempt to determine the agent present in these products which is responsible for the action.

Although the manufacturing procedures in preparing the diastatic enzymes, clarase and taka-diastase, are not known to the writer, it appeared not entirely in order for fungal amylase and mylase P, apparently derived from the same fungus, *Aspergillus oryzae*, to be devoid of a similar neutralizing action upon penicillin. As indicated in the previous paper, it was of interest to note that unlike the ineffectiveness of a malt diastase preparation and particularly of fungal amylase upon penicillin activity, the bacterial amylase used in the study compared in activity with taka-diastase. Upon inquiry concerning the source and possible nature of these two enzyme systems, the following information was obtained from Mr. Philip P. Gray of the Wallerstein Laboratories:

"The fungal amylase preparation is a highly active enzyme preparation obtained from a special strain of *Aspergillus oryzae* by extraction and purification and salt precipitation. This preparation is standardized using sodium sulfate as a diluent to a Lintner value of 1,000 and ordinarily may contain lactose as a carrier. Besides characteristic amylolytic enzymes, the preparation exhibits considerable activity of proteolytic enzymes, cytases, phosphatases, and maltase. Its amylolytic activity is exhibited best at pH range of 4.5 to 5.5 and at temperatures preferably not over 50 C." The mylase P product was described as "representing a specially fractionated mixture of enzymes from the above source (fungal amylase) which will be found to be especially rich in phosphatases, cytases, and hemicellulase, containing also some maltase."

The bacterial amylase preparation, obtained from the same laboratories, was described as "representing an enzyme derived from *Bacillus mesentericus*, a special preparation purified by precipitation and standardized to a definite



starch liquefying strength using sodium chloride, sodium sulfate, and phosphates as diluents. This preparation, while containing proteolytic and other enzymes, contains chiefly *alpha amylase*, exhibits powerful starch liquefying properties, highly active at high temperatures, and at pH values between 6.5 and 8.0."

The purpose of quoting the detailed description of the fungal and bacterial amylase preparations is to correlate the possible common enzyme systems contained in each and to suggest the presence of some enzyme fraction or substance in the bacterial product which may explain the effect of the latter upon penicillin. The active substance is evidently entirely lacking in the fungal amylase or is insufficient in quantity to be demonstrable in the test methods described in the previous communication.

The fact that certain bacterial extracts or enzymes derived from bacterial cells will inactivate penicillin has been known for some time. Fleming (1929) in his early studies on penicillin-containing filtrates observed that the colony-typhoid-dysentery group, as well as *Pseudomonas pyocyaneus*, *Proteus vulgaris*, and *Vibrio cholera*, was resistant or insensitive to penicillin. Abraham and Chain (1940) noted in their studies that an enzyme from *Escherichia coli* extracts destroyed penicillin activity. This enzyme was inactivated by heating at 90 C for 5 minutes and by incubation with papain (activated with potassium cyanide at pH 6.0) and was dialyzable through cellophane membranes. Hobby, Meyer, and Chaffee (1942) also found that supernatant filtrates from *E. coli* cultures inhibited the action of penicillin and noted, furthermore, that the latter agent was inactive against this organism. The author's studies confirm the findings mentioned and add to the list of penicillin-inactivating bacteria the following: *Bacillus subtilis*, *B. mycoides*, *B. megatherium*, *B. mesentericus*, *B. panis*, and *B. adhaerens*; *Serratia marcescens*; and *Clostridium chauvei*. The anaerobic organism mentioned was the only one of a series of pathogenic anaerobes studied which would completely inactivate penicillin. Among the several *Clostridium* cultures used in the investigation which failed to give evidence of a neutralizing effect on penicillin activity were *C. tetani*, *C. novyi*, *C. welchii*, *C. botulinum*, *C. septicum*, *C. sporogenes*, and *C. histolyticum*. *Bacillus brevis*, the aerobic sporulating soil bacillus described by Dubos, which produces tyrothricin, will not inactivate the antibacterial effects of penicillin.

The method used in determining the effects of these and other organisms upon penicillin activity involved the addition of 1 ml of a 24-hour broth culture of organisms, or its sterile filtrate (Berkefeld), to 1 ml of a sodium penicillin solution containing approximately 40 to 48 Oxford units. Following incubation in a water bath at 40 C for two hours, the penicillin-bacterial test solutions were tested for penicillin activity by the Oxford cup-plate method (Abraham *et al.*, 1941).

The information obtained on the neutralizing effects of various bacterial filtrates upon penicillin, particularly organisms of the aerobic, sporeforming *B. subtilis* and associated types, suggested the possibility that similar bacteria or their products might be encountered in the active enzyme preparations, namely, clarase, taka-diaxase, and bacterial amylase, and would account for

the antipenicillin effects. Bacterial counts were made, therefore, on the dry enzyme powders and the organisms present were identified by Mr. G. R. Goetchius, following the description and biochemical reactions given in Bergey's "Manual for Determinative Bacteriology" (5th edition, 1939). This was carried out as follows: Solutions or suspensions of the enzyme preparations were plated in beef extract agar and the colonies developing in the medium were counted at the end of 48 hours' incubation at 37 C. Following these counts, representative colony types were selected and a small inoculum from each was transferred to beef extract broth. At the end of 72 hours' incubation, 4-mm

TABLE 1  
*Bacterial counts and antipenicillin effects of organisms isolated from enzyme preparations*

ENZYME PREPARATIONS	BEEF EXTRACT AGAR PLATE COUNTS ORGANISMS/G	NUMBER COLONY TYPES	NUMBLK IN- ACTIVATING PENICILLIN IN 2 HR	IDENTITY OF ORGANISMS RESPONSIBLE FOR PENICILLIN INACTIVATION
1. Pepsin . . . . .	240,000	2	2	<i>Bacillus teres</i>
2. Trypsin . . . . .	14,000	1	1	<i>B. subtilis</i> group
3. Erepsin . . . . .	4,000	1	1	<i>B. subtilis</i> group
4. Pancreatin . . . . .	23,000	1	1	<i>B. subtilis</i> group
5. Papain . . . . .	21,000	1	none	—
6. Ficin . . . . .	none	—	—	—
7. Hurain . . . . .	none	—	—	—
8. Polidase . . . . .	8,400,000	2	1	<i>B. mesentericus</i>
9. Invertase . . . . .	5,000	1	1	<i>B. subtilis</i> group
10. Malt diastase . . . . .	170,000	3	1	<i>Bacillus cohaerens</i>
11. Mylase "P" . . . . .	99,000	1	1	<i>B. subtilis</i> group
12. Fungal amylase . . . . .	236,000	3	3	<i>B. subtilis</i> group
13. Bacterial amylase . . . . .	48,000,000	3	3	<i>B. subtilis</i> group
14. Clarase "stand." . . . .	180,000,000	2	2	<i>B. subtilis</i> group
15. Clarase "conc." . . . .	398,000,000	4	4	<i>B. subtilis</i> group
16. Taka-diastase . . . . .	13,000,000	2	2	<i>B. subtilis</i> group
17. Emulsin . . . . .	1,000	1	1	<i>B. subtilis</i> group
18. Catalase . . . . .	none	—	—	—
19. Lysozyme . . . . .	10,000,000*	2	1	<i>B. subtilis</i> group

— = no test made.

\* = *Aerobacter aerogenes* predominant organism present.

loopfuls of the bacterial suspension were implanted in Durham tubes containing carbohydrate media as well as in tubes of Bacto-purple milk and nitrate broth. A small sample of the broth culture was also tested at this point for antipenicillin effects by the Oxford cup-plate procedure. The estimated bacterial count per gram of enzyme powder and the colony types along with their identification are given in table 1.

It will be noted from the data that, in general, the total bacterial count varied considerably from one enzyme preparation to another. Furthermore, with but few exceptions, all the samples studied contained organisms which in pure culture were able to inactivate penicillin in less than two hours. Concentrated

clarase gave the highest bacterial count of the products tested, 398,000,000 organisms per gram of dry preparation. Following this, in descending numbers of bacteria per gram of powder, were standardized clarase, bacterial amylase, and taka-diastrase. The numbers of viable organisms estimated in these products were 180,000,000, 48,000,000, and 13,000,000 per gram, respectively. While certain morphological differences were observed in the colony types developing on agar plates, subsequent studies indicated all the strains present in the preparations mentioned could be classified as aerobic, sporeforming *B. subtilis* or related groups.

The predominant organism in the preparation of lysozyme, which contributed to the high count obtained in this preparation, was the gram-negative bacillus, *Aerobacter aerogenes*. This organism failed to show any evidence of an antipenicillin effect when incubated in the presence of the antibiotic agent and tested at the 2-hour period. A *B. subtilis* variant, however, which was capable of inactivating penicillin under the conditions mentioned, was isolated from this enzyme preparation. Polidase was found to contain approximately 8,500,000 bacteria per gram of powder; however, two distinct colony types were isolated from the product, and only one was found to have an antipenicillin effect. The three colony types isolated from plates inoculated with fungal amylase were all identified as belonging to the *B. subtilis* group. The total bacterial count on this preparation, however, was relatively low, i.e., 236,000 bacteria per gram of dry powder.

On the basis of these studies it is evident that the mere presence of penicillin-inactivating organisms in a product does not necessarily indicate that the enzyme preparation will show evidence of an antipenicillin effect. Presence or absence of this action appears to be based upon the numbers of organisms (or possibly their metabolic substances) of the *B. subtilis* group in the products. This correlation in bacterial population with the resulting extent of penicillin inactivation has since been found to hold true for various samples of standardized clarase submitted for this test by the manufacturer. Certain preparations, which were found to be relatively free of bacteria of all types, evidenced little, if any, antipenicillin action.

Standardized clarase preparations were tested, moreover, which gave bacterial counts approximately that of the concentrated clarase sample (table 1, lot 1455) with a corresponding high titer antipenicillin action. The number of bacteria estimated to be present in several standardized clarase samples, and the extent to which freshly prepared sterile filtrates (Berkefeld) of the latter could be diluted and still show evidence of an antipenicillin effect, are given in table 2. These findings were compared with the bacterial counts and antipenicillin titers of the standardized clarase (lot 962) and concentrated clarase (lot 1455) preparations used throughout this and the previous study (Lawrence, 1945).

From the data presented in table 2, it is obvious that there is a correlation between the number of bacteria present in a clarase preparation and the extent to which the sample may be diluted and still show evidence of a neutralizing action against penicillin. Furthermore, the fact that this antipenicillin effect

is demonstrable in sterile Berkefeld filtrates of freshly prepared solutions or suspensions of the various clarase samples would indicate that the presence of viable organisms is not essential for the neutralizing action on the antibiotic agent. On the basis of these findings, therefore, it may be assumed that certain water-soluble, filterable bacterial end products in the sterile filtrates are directly responsible for the inactivation of penicillin noted throughout the studies reported previously (Lawrence, 1943, 1944, 1945).

Reference was made previously to the fact that Abraham and Chain (1940) described an enzyme present in *E. coli* extracts which would destroy the antibacterial effects of penicillin. This enzyme was heat-labile to the extent that it was inactivated by heating at 90 C for 5 minutes. Therefore, the several enzyme systems showing antipenicillin effects in the present study were subjected to the temperature mentioned as well as to 70 C for 20 minutes and also were

TABLE 2  
*Correlation of bacterial count with antipenicillin effect of clarase preparations*

CLARASE PREPARATION	LOT NO.	ESTIMATED BACTERIA PER GRAM OF POWDER	COMPLETE INACTIVATION OF PENICILLIN AT TEST PERIODS (CUP-PLATE)	DILUTION IN FLUID MEDIUM SHOWING ANTIPENICILLIN EFFECT*
Concentrated clarase . . . .	1455	398,000,000	2 hr	1:500,000
Standardized clarase . . . .	962	138,000,000	2 hr	1:350,000
Standardized clarase . . . .	1339	30,000,000	2 hr	1:100,000
Standardized clarase . . . .	1351	135,000,000	2 hr	1:500,000
Standardized clarase . . . .	1426	120,000,000	2 hr	1:500,000
Standardized clarase . . . .	1466	740,000	18 hr	1:2,000
Standardized clarase . . . .	1500	172,000	none	<1:1,000
Standardized clarase . . . .	1526	7,000,000	2 hr	1:20,000

\* = Enzyme and penicillin (app. 42 to 48 Oxford units) contained in 10 ml Brewer's medium. One 4-mm loopful broth culture *Staphylococcus aureus* 209 added to each tube. Results tabulated at end of 7 days' incubation at 37 C. <1:1,000 = concentrations greater than 0.1 per cent not tested.

heated in a boiling water bath. The preparations studied included sterile Berkefeld filtrates of standardized and concentrated clarase, taka-diastrase, and bacterial amylase, and several filtrates of pure cultures of organisms known to show antipenicillin action. A test of the samples against penicillin by the Oxford cup-plate and the broth dilution methods revealed that the active agent present in all the preparations given was destroyed by the heat treatments mentioned.

In earlier articles on this subject (Lawrence, 1944, 1945) mention was made of the fact that in developing the sterility test for penicillin, solutions of the agent were sterilized by heating in a boiling water bath for 3 minutes and cooling rapidly to room temperature. This treatment had no apparent effect upon the potency of the antibiotic solutions.

In the studies presented in this communication, up to this point, the shortest time interval of testing the antibacterial effects of penicillin-enzyme solutions

followed the initial two-hour incubation period at 40 C. With the information available that the antipenicillin agents were more sensitive than penicillin to heat, it appeared worth while to determine the effects of short time intervals of exposure of the antibiotic agent to the active enzyme preparations. This was carried out as follows: To a sample of penicillin solution (containing 42 to 48 Oxford units) was added a sterile filtrate of an active antipenicillin agent. The mixture was incubated in a 40 C water bath and small samples were removed at the end of 5, 10, 15, and 30 minutes and 1, 1½, and 2 hours. Immediately upon removal from the test mixture each sample was placed in a boiling water bath for 3 minutes, following which it was cooled rapidly to room temperature in an ice bath. These solutions, along with suitable controls, were tested for penicillin activity by the Oxford cup-plate procedure. The first sample removed (at the end of 5 minutes) proved to be devoid of any antibacterial effect. The mere contact of an active enzyme solution with penicillin was subsequently shown to be sufficient to suppress or neutralize completely the antibacterial agent. This was proved by adding the enzyme filtrate to a hot (100 C) solution of penicillin in a boiling water bath. Prolonged heating (boiling for 5 to 10 minutes) of an enzyme-penicillin solution, furthermore, did not result in the reappearance of an antibacterial action on the part of the antibiotic substance.

Although Abraham and Chain (1940) found that the antipenicillin enzyme present in *E. coli* filtrates dialyzes through cellophane membranes, the neutralizing substance present in highly active clarase preparations lacks this property completely. The dialytic studies were carried out in distilled water and in buffer solutions at pH 4.0, 6.0, and 8.0. Additional studies are in progress to determine the possible relationship of the antipenicillin agents present in various bacterial filtrates.

#### SUMMARY

Data are presented which indicate that the enzyme preparations that are active against penicillin contain certain water-soluble, Berkefeld-filterable substances of bacterial origin which are responsible for penicillin inactivation.

Broth filtrates of pure cultures of the organisms isolated from the active antipenicillin preparations neutralize the effects of the antibiotic substance. Similar penicillin-inactivating bacteria may be isolated from practically all the enzyme preparations studied.

The presence or absence of an antipenicillin effect in a preparation, including clarase, appears to be based upon the number of organisms (or their metabolic products) which the preparation contains.

The agent or agents present in the active enzyme products which neutralize penicillin activity are heat-labile. The action of active enzyme preparations upon penicillin appears to be almost instantaneous.

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# STUDIES ON THE ACTION OF SULFONAMIDES ON THE RESPIRATION AND GROWTH OF BACTERIA<sup>1</sup>

## A. FACTORS CONTROLLING THE INHIBITION BY SULFONAMIDES OF CARBOXYLASES. I. ANTAGONISM BETWEEN COCARBOXYLASE AND SULFATHIAZOLE

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Studies previously reported on the mode of action of sulfonamides on bacteria showed that the inhibition of respiratory enzymes of the bacteria caused a proportional inhibition of the growth of *Streptococcus pyogenes* and pneumococcus, type I. This conclusion was based on simultaneous measurements, at various time intervals, of the increase in the number of bacteria (and the mg of bacterial nitrogen) and of the respiration in the presence and absence of 0.04 M sulfanilamide. On the basis of these and other observations, the "inhibition of respiration theory," as the mode of action of sulfonamides, was proposed (Sevag and Shelburne, 1942a, 1942b). This theory, in part, stated that chemotherapeutic agents which possess structural similarity to the whole or part of the coenzyme molecules may specifically combine with the protein component of the respiratory enzymes. This combination may take place as a result of the displacement of coenzymes by the drug, forming an inactive "enzyme analogue," or by a reversible union of the drug with the protein, forming an inactive "drug-protein-coenzyme complex." In this connection it was shown (Sevag, Shelburne, and Mudd, 1942) that sulfonamides exercise inhibitory affinities for bacterial and yeast carboxylases. Sulfathiazole, in comparison with other sulfonamides, being structurally most nearly related to cocarboxylase, exercised markedly greater inhibitory affinity for bacterial carboxylases.

The present report represents the results of further studies.

### METHODS

As previously described (Sevag, Shelburne, and Mudd, 1942), carboxylase activities of various materials were measured in a Barcroft-Warburg setup.

*Experiments with air-dried brewers' whole yeast.* The reaction system contained 0.1 ml of a solution of magnesium sulfate (0.1 mg Mg), 0.7 ml of yeast suspension containing various amounts of whole yeast, 0.2 ml of sodium pyruvate (17.6 mg). The final volume was made up to 6 ml with M/150 phosphate buffer of pH 6.2. The temperature of the water bath was 37.5 C. The atmosphere of the system consisted of 95 per cent N and 5 per cent CO<sub>2</sub>, and the experimental period was 120 minutes.

<sup>1</sup> This investigation has been aided by a grant from the Josiah Macy, Jr., Foundation.



*Experiments with yeast washed with alkaline phosphate.* The reaction system contained the same constituents as those given above. In experiment 1 (table 2), the yeast suspension contained 100 mg of air-dried brewers' whole yeast. In experiment 2, 100 mg of yeast were washed with alkaline phosphate, using the method of Lohmann and Schluster (1937) to remove cocarboxylase. The activity of the yeast thus treated was restored by adding pure cocarboxylase (Merck).

## RESULTS

*Reversal by cocarboxylase of the inhibition of the carboxylase activity of yeast cells by sulfathiazole.* The results of two preliminary experiments, presented in table 1, showed that sulfathiazole exercised a 25 per cent inhibition on the carboxylase activity of 2 mg of air-dried yeast cells. This effect was reduced to 14 per cent when 2  $\mu$ g of cocarboxylase was added to the system. This reduction of inhibition is 44 per cent. Two  $\mu$ g of cocarboxylase in a volume of 6 ml is  $6.8 \times 10^{-7}$  M. This concentration of cocarboxylase was found to be capable

TABLE 1

*Reversal by cocarboxylase of the inhibition by sulfathiazole of the carboxylase activity of 2 mg of air-dried brewers' yeast at pH 6.2*

PERIOD	CONTROLS			INHIBITION BY SULFATHIAZOLE (0.0055 M)		
	Buffer	Cocarboxylase		Buffer	Cocarboxylase	
		0.2 $\mu$ g	2.0 $\mu$ g		0.2 $\mu$ g	2.0 $\mu$ g
	mm <sup>3</sup> CO <sub>2</sub>	mm <sup>3</sup> CO <sub>2</sub>	mm <sup>3</sup> CO <sub>2</sub>	per cent	per cent	per cent
1	497	505	618	25	21	14
2	637	690	816	24	26	14

of reducing by 44 per cent the inhibition by 0.0055 M sulfathiazole. That is, one molecule of cocarboxylase caused 44 per cent reversal of the inhibition by 8088 molecules of sulfathiazole.

On washing the whole yeast with alkaline phosphate, 97 per cent of the carboxylase activity was removed (table 2). The yeast, thus treated, contains the specific protein of carboxylase practically free from cocarboxylase. The whole yeast, treated with 3  $\mu$ g, or 50  $\mu$ g, of cocarboxylase, did not show increased carboxylase activity. On the other hand, the treatment of phosphate-washed yeast with 3 or 50  $\mu$ g of cocarboxylase restored 56 and 71 per cent, respectively, of the original carboxylase activity. The specific protein of carboxylase, in washed yeast, treated simultaneously with cocarboxylase and sulfathiazole, should therefore be an object of competition between these two substances.

The results presented in table 2 show that 0.0055 M sulfathiazole did not exercise an inhibitory effect on 100 mg of whole yeast. In contrast, it exercised a 35 per cent inhibition on the carboxylase activity of washed cells in the presence of 3  $\mu$ g of added cocarboxylase. In the presence of 50  $\mu$ g of cocarboxylase the inhibition was reduced to 5 per cent, or an 86 per cent reversal of inhibition

took place. This relationship shows that one molecule of cocarboxylase is capable of preventing the union of 322 molecules of sulfathiazole with the specific carboxylase protein.

TABLE 2

*Reversal by cocarboxylase of the inhibition of the carboxylase activity of yeast (washed with alkaline phosphate) by 0.0055 M sulfathiazole*

ENZYME SYSTEM	CONTROL*			INHIBITION BY SULFATHIAZOLE		
	Buffer	Cocarboxylase		Buffer	Cocarboxylase	
		3 $\mu$ g	50 $\mu$ g		3 $\mu$ g	50 $\mu$ g
	mm <sup>3</sup> CO <sub>2</sub>	mm <sup>3</sup> CO <sub>2</sub>	mm <sup>3</sup> CO <sub>2</sub>	per cent	per cent	per cent
1. Brewers' whole yeast (100 mg air-dried)	2,172	2,182	2,200	0	0	-9
2. Brewers' whole yeast† (100 mg washed with alkaline phosphate)	61	1,215	1,556	10	35	5

- = increase.

\* Control system consisted of 0.1 ml of MgSO<sub>4</sub> (0.1 mg of Mg) solution + 0.7 ml of yeast suspension + 0.2 ml of sodium pyruvate (17.6 mg), made up to a volume of 6 ml with M/150 phosphate buffer of pH 6.2. Temperature = 37.5 C. In 95% N + 5% CO<sub>2</sub>. Period = 120 minutes.

† Air-dried yeast was washed with alkaline phosphate according to Lohmann and Schuster (1937) to remove cocarboxylase.

TABLE 3

*Reversal by cocarboxylase of the inhibition of the carboxylase activity of both the whole air-dried brewers' yeast (10 mg) and that of yeast treated with alkaline phosphate by 0.0055 M sulfathiazole*

ENZYME SYSTEM	CONTROLS				INHIBITION BY SULFATHIAZOLE			
	Buffer	Cocarboxylase $\mu$ g			Buffer	Cocarboxylase $\mu$ g		
		0.3	3	25		0.3	3	25
	mm <sup>3</sup> CO <sub>2</sub>	mm <sup>3</sup> CO <sub>2</sub>	mm <sup>3</sup> CO <sub>2</sub>	mm <sup>3</sup> CO <sub>2</sub>	per cent	per cent	per cent	per cent
1. Brewers' whole yeast (10 mg air-dried)	1,280	1,330	1,435	1,520	20	-8	0	-1
2. Brewers' yeast washed with alkaline phosphate*	22	117	683	1,150	90	80	80	56†

- = increase.

\* For experimental conditions see the first footnote table 2.

† This represents the inhibition at the end of a 2-hour period. At the end of the initial 30-minute period the inhibition was 67 per cent. The inhibitions not indicated in the preceding columns were constant throughout the 2-hour period.

Another experiment (table 3), carried out with 10 mg of whole air-dried yeast, or with the yeast washed with alkaline phosphate, gave the same results. In these experiments, 3 and 25  $\mu$ g of cocarboxylase increased the carboxylase activity 12 and 19 per cent, respectively. In the absence of added cocarboxylase, 0.0055 M sulfathiazole exercised a 20 per cent inhibition. In the presence of

0.3  $\mu\text{g}$  of cocarboxylase this inhibition was abolished. This means that one molecule of cocarboxylase was capable of neutralizing the inhibitory effect of 53,400 molecules of sulfathiazole.

TABLE 4

*Effect of the presence and absence of glucose in culture medium on the carboxylase activity of Staphylococcus aureus*

	CARBOXYLASE ACTIVITY OF WASHED SUSPENSIONS OF STAPHYLOCOCCUS AUREUS GROWN IN			
	Seeded with culture on extract agar		Seeded with culture on glucose agar	
	Extract* broth	Glucose broth	Glucose† broth	Extract broth
Carboxylase Activity at pH.....	7.2	7.2	7.2	7.2
Period of experiment.....	4 hr	5 hr	4 hr	5 hr
mm <sup>3</sup> CO <sub>2</sub> evolved.....	683	0	0	643
QCO <sub>2</sub> ‡....	216	0	0	163

\* Extract broth consisted of 10 g of peptone, 5 g of sodium chloride, and 3 g of beef extract (Difco) in 1,000 ml of tap water, boiled, filtered, adjusted to pH 7.4, and sterilized. Extract agar consisted of extract broth containing 2 per cent agar.

† Glucose broth consisted of the same materials as above except that sodium chloride was replaced by 4 g of anhydrous disodium phosphate per liter of medium and adjusted to pH 7.4. To the sterilized medium a concentrated sterile solution of glucose was added just before seeding the medium (final concentration, 0.5% glucose in the medium).

Glucose agar consisted of glucose broth containing 2 per cent agar.

‡ mm<sup>3</sup> CO<sub>2</sub> evolved per hour per mg of staphylococci.

TABLE 5

*Effect of pH on the degree of inhibition exercised by sulfathiazole on the carboxylase activity of Staphylococcus aureus*

PERIOD  minutes	pH 7.16			pH 6.2		
	Control* QCO <sub>2</sub> †	Inhibition by sulfathiazole		Control† QCO <sub>2</sub>	Inhibition by sulfathiazole	
		0.00138 M	0.0055 M		0.00138 M	0.0055 M
		per cent	per cent		per cent	per cent
30	68	36	60	104	14	31
60	90	35	61	125	16	30
90	101	31	60	134	14	29
120	114	29	58	146	13	29

\* The reaction system (pH 7.16) contained 3.1 mg of *S. aureus*.

† The reaction system (pH 6.2) contained 2.1 mg of *S. aureus*.

‡ mm<sup>3</sup> CO<sub>2</sub> evolved per hour per mg of staphylococci.

The bacterial suspensions used in the two sets of experiments were prepared from the same 16-hour broth culture.

Washing 10 mg of yeast with alkaline phosphate removed 98 per cent of the carboxylase activity. The treatment of the washed yeast with 25  $\mu\text{g}$  of cocarboxylase restored 76 per cent of the original activity. In the presence of 0.0055 M sulfathiazole and 0.3 or 3  $\mu\text{g}$  of cocarboxylase, 80 per cent of the restorable

activity was inhibited. On the other hand, in the presence of 25  $\mu\text{g}$  of cocarboxylase the inhibition was reduced from 80 to 56 per cent. This is equal to a 30 per cent reversal of inhibition. In other words, one molecule of cocarboxylase counteracted 646 molecules of sulfathiazole in bringing about this effect.

*Experiments on the carboxylase activity of Staphylococcus aureus.* During our daily studies, extending over a period of two years, the gradual decrease and eventual loss of carboxylase activity in *Staphylococcus aureus* was observed. It has been previously noticed that certain strains were completely devoid of carboxylase activity (Sevag and Neue-Schwander-Lemmer, 1936). Krebs (1937) also reported similar observations. The causes of these variations are not known. During our studies it has been possible to trace one of the factors responsible for the complete loss of carboxylase activity. The results presented

TABLE 6

*Reversal by cocarboxylase of the inhibition by sulfathiazole (0.00138 M) of the carboxylase activity of Staphylococcus aureus*

The pH of the reaction system was 6.2; the weight of staphylococci was 3 mg/6 ml reaction volume.

EXP NO.	PERIOD	CONTROLS				INHIBITION BY SULFATHIAZOLE			
		Buffer	Cocarboxylase $\mu\text{g}$			Buffer	Cocarboxylase $\mu\text{g}$		
			5	10	25		5	10	25
	minutes	mm <sup>3</sup> CO <sub>2</sub>	mm <sup>3</sup> CO <sub>2</sub>	mm <sup>3</sup> CO <sub>2</sub>	mm <sup>3</sup> CO <sub>2</sub>	per cent	per cent	per cent	per cent
1	30	76	122	120	116	30	30	24	16
	60	167	252	250	233	25	21	18	13
	90	253	377	382	351	25	23	20	11
	120	339	489	486	458	22	18	12	10
2	30	217	249	268	222	28	18	23	14
	60	470	556	594	482	26	20	23	11
	90	735	950	900	735	27	30	23	12
	120	978	1148	1170	970	25	22	19	9

in table 4 are related to this phenomenon. It can be seen that staphylococci, when grown in glucose phosphate extract broth, were completely devoid of carboxylase activity. In contrast, when they were grown in plain extract broth, they showed carboxylase activity. This observation, though made several times, cannot, however, at present be offered as proof that growth in glucose always deprives the organism of carboxylase activity. Further studies are in progress.

In consideration of the importance of the optimal carboxylase activity, and of the optimal inhibitory effect of sulfathiazole, experiments were carried out at pH 7.16 and pH 6.2. The results presented in table 5 show that the carboxylase activity of *Staphylococcus aureus* at pH 6.2 is markedly greater than at pH 7.16. It can also be seen that the inhibitory effect of sulfathiazole at pH 6.2 is lower by 50 to 60 per cent than at pH 7.16.

In view of the fact that carboxylase activity is optimal at pH 6.2, and that its

sulfonamide-antagonizing action can best be observed at its optimal pH of activity, the experiments were carried out at this pH.

The results presented in table 6 (experiment 1) show that 5  $\mu$ g (also 10 and 25  $\mu$ g) of cocarboxylase increased the carboxylase activity of staphylococci 50 (one-hour period) and 44 (two-hour period) per cent. The results of experiment 2 show a similar effect. In the absence of cocarboxylase, sulfathiazole causes from 18 to 30 per cent inhibition of activity. The addition of 25  $\mu$ g cocarboxylase to the system causes from 50 to 65 per cent reversal of inhibition. This indicates that one molecule of cocarboxylase is capable of counteracting 215 molecules of sulfathiazole in bringing about this effect.

#### SUMMARY

Sulfathiazole inhibits the carboxylase activity of whole yeast. It is shown that one molecule of cocarboxylase added to the reaction system is capable of counteracting the inhibitory effect of 8,088 to 53,400 molecules of sulfathiazole.

Washing yeast cells with alkaline phosphate removes practically all of the carboxylase activity. Addition of cocarboxylase restores from 56 to 76 per cent of the original activity. Under these conditions sulfathiazole and cocarboxylase compete for the specific carboxylase protein. This competition results in the neutralization of the inhibition by sulfathiazole. One molecule of cocarboxylase counteracts the inhibition exercised by 322 to 646 molecules of sulfathiazole.

Sulfathiazole inhibits the carboxylase activity of *Staphylococcus aureus*. One molecule of cocarboxylase counteracts the inhibitory effect of 215 molecules of sulfathiazole.

Staphylococci when grown in glucose phosphate extract broth have been found to be devoid of carboxylase activity. Staphylococci when grown in glucose-free plain extract broth have been found to manifest good carboxylase activity. The reason for this is not known.

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# STUDIES ON THE ACTION OF SULFONAMIDES ON THE RESPIRATION AND GROWTH OF BACTERIA<sup>1</sup>

## A. FACTORS CONTROLLING THE INHIBITION BY SULFONAMIDES OF CARBOXYLASES. II. ANTAGONISM BETWEEN *p*-AMINOBENZOIC ACID AND SULFATHIAZOLE

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In a preceding report it was shown that cocarboxylase antagonizes the inhibitory effect exercised by sulfathiazole on the carboxylase activities of (a) whole yeast cells, (b) yeast cells washed with alkaline phosphate, and (c) *Staphylococcus aureus*. The results showed that one molecule of cocarboxylase counteracted the inhibitory effect of 322 to 53,400 molecules of sulfathiazole on yeast carboxylase, and the inhibitory effect of 215 molecules of sulfathiazole on *Staphylococcus aureus*. The present report deals with experiments in which the antagonism between sulfathiazole and *p*-aminobenzoic acid is determined.

### RESULTS

*Competition between sodium pyruvate and sulfathiazole for the carboxylase of Escherichia coli.* To determine the exact mechanism of the action of sulfathiazole on the carboxylase activity of bacteria, the following experiments were conducted. In one experiment, using two-armed Warburg flasks, sulfathiazole was first mixed with the bacteria, and then the substrate, sodium pyruvate, was added. In another experiment, sulfathiazole was added to the system immediately after sodium pyruvate and the bacteria were mixed. The results of these two simultaneous experiments are given in table 1. It can be seen that when sodium pyruvate was added to the mixture of sulfathiazole and bacteria, the carboxylase activity was inhibited from 35 to 37 per cent. In contrast, when sulfathiazole was added to the mixture of sodium pyruvate and bacteria, the inhibition was only from 4 to 7 per cent. This shows that both the substrate and sulfathiazole are attracted to the same active sites in the enzyme carboxylase.

*Failure of p-aminobenzoic acid to reverse the inhibition by acetaldehyde of the carboxylase activity of E. coli.* It is known that acetaldehyde, the decarboxylation product of sodium pyruvate, exercises a strong inhibitory effect on the carboxylase activity of cells. This inhibitor evidently combines specifically with the active site of the enzyme and prevents its activity. This could be compared with the inhibition of carboxylase activity by sulfathiazole if both inhibitions could be counteracted by *p*-aminobenzoic acid. It was therefore of interest

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to determine whether or not the inhibition by acetaldehyde is reversible by *p*-aminobenzoic acid. The results of experiments are presented in table 2.

It can be seen that  $4.06 \times 10^{-2}$  M of acetaldehyde alone caused 83 per cent inhibition of the carboxylase activity of *E. coli*;  $1 \times 10^{-2}$  M *p*-aminobenzoic acid caused a 30 per cent, and  $2.5 \times 10^{-3}$  M, a 17 per cent inhibition. Also  $1 \times 10^{-2}$  M,  $2.5 \times 10^{-3}$  M, and  $6.25 \times 10^{-4}$  M *p*-aminobenzoic acid failed to

TABLE 1

*Competition between sodium pyruvate ( $5 \times 10^{-2}$  M) and sulfathiazole ( $4.14 \times 10^{-3}$  M) for the carboxylase of *E. coli* (1.238 mg)*

The pH of the reaction medium was 7.2

E. COLI	SODIUM PYRUVATE ADDED LAST				SULFATHIAZOLE ADDED LAST			
	Control		Sulfathiazole		Control		Sulfathiazole	
	a*	b*	a	b	a	b	a	b
mm <sup>3</sup> CO <sub>2</sub> evolved (2 hr).....	488	520	318	324	406	413	390	380
QCO <sub>2</sub> †.....	197	210	128	131	164	166	157	153
Per cent inhibition.....	—	—	35	37	—	—	4	7

\* a and b represent the results of two different experiments.

† QCO<sub>2</sub> = mm<sup>3</sup> CO<sub>2</sub>/hour/mg *E. coli*.

TABLE 2

*Failure of p-amino benzoic acid to reverse the inhibition of the carboxylase activity of *E. coli* (1.088 mg) by acetaldehyde ( $4.06 \times 10^{-2}$  M) and vice versa*

E. COLI	CONTROL		p-AMINO BENZOIC ACID					
			$1 \times 10^{-2}$ M		$2.5 \times 10^{-3}$ M		$6.25 \times 10^{-4}$ M	
	Buffer	Acet-alde-hyde	Buffer	Acet-alde-hyde	Buffer	Acet-alde-hyde	Buffer	Acet-alde-hyde
mm <sup>3</sup> CO <sub>2</sub> evolved (3 hr).....	882	132	593	51	706	92	781	131
QCO <sub>2</sub> *.....	261	44	182	15	216	27	240	36
Per cent inhibition (when compared with absolute control)....	—	83	30	94	17	90	8	87
Per cent inhibition (when compared with the respective controls) ...	—	83	30	92	17	87	8	85

\* QCO<sub>2</sub> = mm<sup>3</sup> of CO<sub>2</sub>/hour/mg *E. coli*.

exercise any antagonistic action on the inhibition by  $4.06 \times 10^{-2}$  M acetaldehyde. In the presence of both acetaldehyde and *p*-aminobenzoic acid the inhibition was the same as that exercised by acetaldehyde alone. That is, the inhibition in the presence of both substances was not additive.

*Reversal by p-aminobenzoic acid of the inhibition by sulfathiazole of the carboxylase activity of E. coli.* It was shown above that *p*-aminobenzoic acid was incapable of counteracting the inhibition exercised on the carboxylase activity of *E. coli* by acetaldehyde. In the following experiments acetaldehyde was replaced by sulfathiazole. The results are presented in table 3. It can be seen

TABLE 3

Reversal by *p*-aminobenzoic acid (*p*-ABA) of the inhibition by  $2.8 \times 10^{-3}M$  sulfathiazole of the carboxylase activity of *E. coli*

PERIOD	CONTROL*	INHIBITION BY SULFATHIAZOLE	INHIBITION BY		REVERSAL BY <i>p</i> -ABA ( $6.7 \times 10^{-4}M$ ) OF THE INHIBITION BY SULFATHIAZOLE	INHIBITION BY		REVERSAL BY <i>p</i> -ABA ( $6.7 \times 10^{-3}M$ ) OF THE INHIBITION BY SULFATHIAZOLE
			<i>p</i> -ABA ( $6.7 \times 10^{-4}M$ ) alone	<i>p</i> -ABA + sulfathiazole		<i>p</i> -ABA ( $6.7 \times 10^{-3}M$ ) alone	<i>p</i> -ABA + sulfathiazole	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
minutes	mm <sup>3</sup> CO <sub>2</sub>	per cent	per cent	per cent	per cent	per cent	per cent	per cent
30	94	26	6	0	100†	22	26	85‡
60	317	19	6	5	100	21	20	100
120	810	20	6	10	80	21	19	100

\* The reaction system consisted of 0.2 ml of *E. coli* (measured with a Kahn pipette) suspension in M/15 phosphate buffer of pH 7.16 + 5.4 ml of M/120 phosphate buffer of pH 7.16 + 0.1 ml of MgSO<sub>4</sub> solution (0.1 mg Mg) + 0.3 ml of sodium pyruvate of pH 7.16. Temperature = 37.5 C. Atmosphere = 95% N + 5% CO<sub>2</sub>.

† The percentage of reversal was calculated by comparing the differences between columns (5) and (4) with that given under column (3), or column (3) - [column (5) - (4)]/(3) × 100 = % reversal.

‡ The values were obtained by identical treatment as described under the preceding note.

TABLE 4

Reversal by *p*-aminobenzoic acid (*p*-ABA) of the inhibition of the carboxylase activity of *Escherichia coli* (2.016 mg) by  $2.8 \times 10^{-3}M$  sulfathiazole at pH 7.2

E. COLI	CON- TROL (BUF- FER)	SULFA- THIA- ZOLE	<i>p</i> -AMINO BENZOIC ACID			
			$6.2 \times 10^{-3}M$		$2.5 \times 10^{-4}M$	
			Buffer	Sulfa- thiazole	Buffer	Sulfa- thiazole
	a	b	a'	b'	a'	b'
mm <sup>3</sup> CO <sub>2</sub> evolved (3 hr).....	1205	735	950	694	844	765
QCO <sub>2</sub> .....	200	121	157	114	138	126
Inhibition, per cent						
a. When compared with absolute control. ....	—	40	21	45	31	37
b. When compared with respective controls. ....	—	40	21	27	31	8
Reversal by <i>p</i> -ABA of the inhibition by sulfathiazole, in per cent.....	—	—	—	32*	—	80

\*  $[a-b/a] \times 100 = A$ , per cent inhibition by sulfathiazole.

$[a'b'/a'] \times 100 = B$ , per cent inhibition by sulfathiazole in the presence of *p*-ABA. (This is on the assumption that the inhibition by *p*-ABA is unchanged by addition of sulfathiazole.)

$A-B/A \times 100 =$  per cent reversal by *p*-ABA of the inhibition by sulfathiazole.

that  $6.7 \times 10^{-4}M$  *p*-aminobenzoic acid alone caused only a 6 per cent inhibition, and  $6.7 \times 10^{-3}M$  caused 22 per cent inhibition of carboxylase activity. On the other hand,  $2.8 \times 10^{-3}M$  sulfathiazole alone caused from 19 to 26 per cent



inhibition. This inhibition is reversed from 80 to 100 per cent by  $6.7 \times 10^{-4}$  M, or by  $6.7 \times 10^{-3}$  M *p*-aminobenzoic acid. In the simultaneous presence of  $6.7 \times 10^{-3}$  M *p*-ABA and  $2.8 \times 10^{-3}$  M ST the inhibition of carboxylase activity was equal to that caused by either alone. This indicates that the inhibition is not additive, and that both substances compete for the same active group in the enzyme. This may be interpreted to indicate that *p*-ABA antagonizes by maintaining its own inhibitory effect (when a higher concentration is used) on the enzyme and thus preventing sulfathiazole from exercising its inhibitory effect. In a lower concentration ( $6.7 \times 10^{-4}$  M), *p*-ABA maintains its own union with the enzyme, without exercising inhibitory effect, and thus also prevents sulfathiazole from exercising an inhibitory effect. In the former case one molecule of *p*-ABA antagonized about 2 molecules of ST. In the latter case one molecule of *p*-ABA antagonized about 20 molecules of ST.

TABLE 5

*Reversal by p-aminobenzoic acid of the inhibition by sulfathiazole of the carboxylase activity of Staphylococcus aureus*

PERIOD	QCO <sub>2</sub> —CONTROL			INHIBITION BY SULFATHIAZOLE $1.38 \times 10^{-3}$ M		
	Buffer	<i>p</i> -Aminobenzoic acid		Buffer	<i>p</i> -Aminobenzoic acid	
		$1 \times 10^{-4}$ M	$1.2 \times 10^{-2}$ M		$1.4 \times 10^{-4}$ M	$1.2 \times 10^{-2}$ M
hr				per cent	per cent	per cent
1	55	52	75	40	32	26
2	71	69	84	45	32	24
3	78	74	85	42	27	21
4	80	80	86	38	28	18
5	80	80	85	33	25	17

The reaction system consisted of 0.2 ml of staphylococcal (2 mg) suspension + 0.1 ml of MgSO<sub>4</sub> sol. (0.1 mg of Mg) + 5.4 ml of M/150 phosphate buffer of pH 7.16 + 0.3 ml of sodium pyruvate (25 mg) in an atmosphere of 95 per cent N and 5 per cent CO<sub>2</sub>, at a temperature of 37.5 C. QCO<sub>2</sub> = mm<sup>3</sup> CO<sub>2</sub>/hour/mg staphylococci.

In a similar experiment (table 4),  $6.2 \times 10^{-5}$  M and  $2.5 \times 10^{-4}$  M *p*-ABA alone caused, respectively, 21 and 31 per cent inhibition of carboxylase activity;  $2.8 \times 10^{-3}$  M ST alone caused 40 per cent inhibition. In the simultaneous presence of *p*-ABA and ST, the inhibition was not only not additive but with  $2.5 \times 10^{-4}$  M *p*-ABA the 40 per cent inhibition by ST was reduced to 8 per cent, and with  $6.2 \times 10^{-5}$  M *p*-ABA it was reduced to 27 per cent. These amount to from 32 to 80 per cent reversal of inhibition. In bringing about an 80 per cent reversal of inhibition, one molecule of *p*-ABA was capable of antagonizing 10 molecules of ST.

*Reversal by p-aminobenzoic acid of the inhibition by sulfathiazole of the carboxylase activity of Staphylococcus aureus.* The results of an experiment with staphylococci are presented in table 5. In this experiment the hourly course of the antagonism between *p*-ABA and ST was determined for a period of five hours. It can be seen that in the presence of  $1.4 \times 10^{-4}$  M *p*-ABA the 33 to 45 per cent

inhibition by  $1.38 \times 10^{-3}$  M ST was reduced to 25 to 32 per cent, and with  $1.2 \times 10^{-3}$  M *p*-ABA the inhibitions were reduced to from 17 to 26 per cent. These amount to a reversal of inhibition of from 35 to 53 per cent by ST. That is, one molecule of *p*-ABA antagonized, respectively, 10 and 1 molecules of ST.

#### DISCUSSION

*p*-Aminobenzoic acid was found to be incapable of reversing the inhibition, exercised on the carboxylase activity of *E. coli*, by acetaldehyde. The inhibition in the simultaneous presence of *p*-aminobenzoic acid and acetaldehyde was found to be nonadditive despite the fact that each alone caused, respectively, 83 and 30 per cent inhibition. In the presence of both, the observed inhibition corresponded to that exercised by acetaldehyde alone. Evidently the inhibitory combination between acetaldehyde and carboxylase prevents *p*-aminobenzoic acid from also combining with the active site of the enzyme. In contrast, *p*-aminobenzoic acid counteracted the inhibition exercised on carboxylase by sulfathiazole. One molecule of the former was capable of counteracting from 1 to 20 molecules of the latter. In a preceding report it was demonstrated that one molecule of cocarboxylase was capable of counteracting the inhibitory action of from 200 to 600 molecules of sulfathiazole on the activity of carboxylase. These show that the coenzyme cocarboxylase is from 10- to 20-fold more effective as a sulfathiazole antagonist than is *p*-aminobenzoic acid.

In this connection a reference to previous observations may be of interest. In experiments extending over a 4-hour period, Sevag and Shelburne (1942) found that one molecule of *p*-aminobenzoic acid neutralized the inhibitory effect exercised on the aerobic and anaerobic respiration of *Streptococcus pyogenes* by 6 to 66 molecules of sulfanilamide. Clifton and Loewinger (1943) reported that the inhibitory effect of sulfanilamide on the anaerobic respiration of *E. coli* is prevented by *p*-aminobenzoic acid. Their results show that one molecule of *p*-aminobenzoic acid neutralized 125 molecules of sulfanilamide.

In connection with the results on the inhibition of carboxylase by sulfathiazole, it may also be of interest to refer to the fact that one molecule of *p*-aminobenzoic acid was found by Wyss *et al.* (1942) to neutralize the inhibition of the half-maximal growth (16-hour period) of *Staphylococcus aureus* by 53, and that of *E. coli* by 27 molecules of sulfathiazole. These ratios compare favorably with those found in our experiments on the carboxylase activity of *S. aureus* and *E. coli*.

In all of these instances, there is no evidence that *p*-aminobenzoic acid participates actively in the metabolic activities of the organisms studied to account for its sulfonamide-antagonizing property. This is particularly true in those cases in which resting cells, and isolated enzyme systems, have been tested. Kohn and Harris (1941) postulated that *p*-aminobenzoic acid acts as a catalyst. As will be discussed later, this is contrary to the known facts. It is known that *p*-aminobenzoic acid inhibits the growth of certain bacteria and the activity of certain enzymes. This will be evident also from the discussions which follow. Considering, however, the postulate of Kohn and Harris in the light of the



# STUDIES ON THE ACTION OF SULFONAMIDES ON THE RESPIRATION AND GROWTH OF BACTERIA<sup>1</sup>

## A. FACTORS CONTROLLING THE INHIBITION BY SULFONAMIDES OF CARBOXYLASES. III. ANTAGONISM BETWEEN NEOPEPTONE AND SERUM PROTEINS, AND SULFONAMIDES

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In two previous reports, it was shown that cocarboxylase and *p*-aminobenzoic acid counteract the inhibitory effect of sulfathiazole on yeast and bacterial carboxylases. Whereas cocarboxylase brings about this effect without exercising any inhibitory action on carboxylase, *p*-aminobenzoic acid exercises the antagonistic action to sulfathiazole while maintaining a certain degree of inhibitory effect of its own on carboxylases. That is, *p*-aminobenzoic acid behaves as an inhibitor and thereby counteracts the effect of other inhibitors weaker or stronger than itself.

In the preceding studies, the experimental conditions were such that cells, exercising carboxylase activity, could not multiply. In the present study, the antagonism between proteins and sulfonamides was investigated. Under these conditions cells multiplied; and growth was estimated from measurements of turbidity with the Klett-Summerson photoelectric colorimeter. To correlate the weight of cells with their carboxylase activity at a given time, the bacterial turbidity was measured immediately after the last manometric reading. To obtain approximate values, the averages of initial and final numerical values were calculated. They are given in the following tables. These, no doubt, do not represent absolute values. However, since, in a study of this nature, only the comparative values are of significance, the expressed average results do not involve errors of a serious nature.

### RESULTS

#### *Reversal by neopeptone of the inhibition of carboxylase activity of Staphylococcus aureus and Escherichia coli by sulfathiazole*

The results presented in table 1 with *Staphylococcus aureus* show that in a buffer mixture the carboxylase activity of staphylococci is inhibited 63 per cent by  $4.14 \times 10^{-3}$  M sulfathiazole. This inhibition is reduced to 35 and 20 per cent, respectively, by 0.043 and 0.172 per cent neopeptone. This corresponds to from 44 to 68 per cent reversal of inhibition.

The results of experiments with *Escherichia coli* are presented in table 2. It can be seen that  $4.14 \times 10^{-3}$  M sulfathiazole exercises a 57 per cent inhibition

<sup>1</sup> This investigation has been aided by a grant from the Josiah Macy, Jr., Foundation.

of carboxylase activity. In the presence of 0.086 and 0.86 per cent neopeptone this inhibition was reduced to 32 and 15 per cent, respectively.

TABLE 1

*Reversal by neopeptone of the inhibition of the carboxylase activity of Staphylococcus aureus by sulfathiazole*

STAPHYLOCOCCUS AUREUS	CONTROLS				SULFATHIAZOLE, $4.14 \times 10^{-2}$ M			
	Buffer	Neopeptone (per cent)			Buffer	Neopeptone (per cent)		
		0.043	0.172	0.86		0.043	0.172	0.86
Weight of staphylococci in mg ( $3\frac{1}{2}$ -hr period) . . . . .	2.08	2.39	2.59	2.75	2.08	2.39	2.59	2.75
mm <sup>3</sup> CO <sub>2</sub> evolved ( $3\frac{1}{2}$ -hr period) . . . . .	772	874	883	880	286	570	710	705
QCO <sub>2</sub> . . . . .	106	104	97	90	39	68	78	72
Inhibition (per cent) . . . . .	—	—	—	—	63	35	20	20

\* Weight of inoculum was 2.08 mg of staphylococci. QCO<sub>2</sub> = mm<sup>3</sup> CO<sub>2</sub>/hour/mg staphylococci.

TABLE 2

*Reversal by neopeptone of the inhibition of the carboxylase activity of Escherichia coli by  $4.14 \times 10^{-2}$  M sulfathiazole (ST)*

E. COLI	BUFFER		NEOPEPTONE 0.086%		NEOPEPTONE 0.86%	
	Control	ST	Control	ST	Control	ST
<i>E. coli</i> in mg (4-hr period) . . . . .	0.58	0.58	0.72	0.63	0.79	0.74
mm <sup>3</sup> CO <sub>2</sub> (4-hr period) . . . . .	268	117	943	562	1,666	1,320
QCO <sub>2</sub> . . . . .	116	50	328	223	526	447
Inhibition (per cent) . . . . .	—	57	—	32	—	15

QCO<sub>2</sub> = mm<sup>3</sup> CO<sub>2</sub>/hour/mg *E. coli*.

*Comparison of the effects exercised by neopeptone and serum albumin in counteracting the inhibition of the carboxylase activity of E. coli by para-, meta-, and ortho-aminobenzenesulfonamides,<sup>2</sup> and para-aminobenzoic acid*

The results presented in table 3 show that in simple buffer medium *para*-, *meta*-, and *ortho*-aminobenzenesulfonamides inhibited the carboxylase activity of *E. coli*, respectively, 68, 43, and 100 per cent. In the presence of 0.86 per cent neopeptone the inhibitions were reduced, respectively, to 19, 7, and 10 per cent. In contrast, neopeptone did not exercise any antagonism to the inhibition by *p*-aminobenzoic acid. This indicates that this substance exercises a greater affinity for carboxylase than for neopeptone.

The results of experiments with human serum albumin (from Dr. H. B.

<sup>2</sup> We are indebted to Dr. R. O. Roblin, Jr., of the American Cyanamid Company, and to Dr. R. H. Kienle, of the Calco Chemical Division of the American Cyanamid Company, for the gift of the samples of *meta*- and *ortho*-aminobenzenesulfonamides.

Vickery) are presented in table 4. It can be seen that in simple buffer medium *para*-, *meta*-, and *ortho*-aminobenzenesulfonamides inhibit the carboxylase activity, respectively, 71, 25, and 82 per cent. In the presence of 2 per cent serum albumin, the inhibition by the *para* isomer showed no change. With the *meta* isomer the inhibition was not only abolished, but the activity was increased 40 per cent, and 82 per cent inhibition by the *ortho* isomer was reduced to 8 per

TABLE 3

*Reversal by ncopeptone (0.86 per cent) of the inhibition of the carboxylase activity of E. coli by  $2.4 \times 10^{-2} M$  para-, meta-, and ortho-aminobenzenesulfonamides, and para-aminobenzoic acid*

E. COLI	CONTROL		PARA		META		ORTHO		p-ABA	
	Buffer	Neo-peptone	Buffer	Neo-peptone	Buffer	Neo-peptone	Buffer	Neo-peptone	Buffer	Neo-peptone
<i>E. coli</i> in mg (4 hr) . . . .	0.54	0.76	0.54	0.76	0.54	0.76	0.54	0.74	0.54	0.68
mm <sup>3</sup> CO <sub>2</sub> (4 hr) . . . .	415	1,992	127	1,610	235	1,850	0	1,748	129	239
QCO <sub>2</sub> . . . . .	192	659	59	533	109	612	0	592	60	87
Inhibition (per cent) . .	—	—	68	19	43	7	100	10	68	87

QCO<sub>2</sub> = mm<sup>3</sup> CO<sub>2</sub>/hour/mg *E. coli*.

TABLE 4

*Comparison of the inhibitory effects of  $2.4 \times 10^{-2} M$  para-, meta-, and ortho-aminobenzenesulfonamides and para-aminobenzoic acid on the carboxylase activity of E. coli in the presence of serum albumin (2 per cent)*

E. COLI	CONTROL		PARA		META		ORTHO		p-ABA	
	Buffer	Al-bumin	Buffer	Al-bumin	Buffer	Al-bumin	Buffer	Al-bumin	Buffer	Al-bumin
<i>E. coli</i> in mg (2 hr) . . . . .	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83
mm <sup>3</sup> CO <sub>2</sub> (2 hr) . . . . .	162	61	48	17	123	85	29	56	68	6
QCO <sub>2</sub> . . . . .	100	37	29	10	75	52	18	34	41	3
Inhibition (per cent) . . . . .	—	63	71	73	25	-40	82	8	59	92

QCO<sub>2</sub> = mm<sup>3</sup> CO<sub>2</sub>/hour/mg *E. coli*.

cent. The inhibition by *p*-aminobenzoic acid in buffer medium was 59 per cent, and in serum albumin it increased to 92 per cent. It is of interest that both *para*-aminobenzenesulfonamide and *p*-aminobenzoic acid, in contrast to *meta*- and *ortho*-aminobenzenesulfonamides, maintain their inhibitory effects in the presence of serum albumin. It must also be noted that 2 per cent serum albumin itself causes a marked inhibition of carboxylase activity. In this respect, and in its antagonistic action, human serum albumin behaves similarly to *p*-aminobenzoic acid.

*Reversal by neopeptone of the inhibition of carboxylase activity of E. coli by serum globulin*

It was shown above that fractionated human serum albumin exercises an inhibitory effect on the carboxylase activity of *E. coli*. The results presented in table 5 show also that 0.017 and 0.085 per cent fractionated human serum globulin caused, respectively, 35 and 68 per cent inhibition of carboxylase activity. In the presence of 0.085 per cent neopeptone these inhibitions were reduced to 8 and 48 per cent respectively. These reductions correspond to 77 and 30 per cent reversal of inhibitions.

TABLE 5

*Reversal by neopeptone (0.085 per cent) of the carboxylase activity of Escherichia coli by serum globulin*

E. COLI	CONTROLS		GLOBULIN (0.017%)		GLOBULIN (0.085%)	
	Buffer	Neo-peptone	Buffer	Neo-peptone	Buffer	Neo-peptone
<i>E. coli</i> in mg (4 hr) . . . . .	0.83	1.24	0.83	1.24	0.82	1.24
mm <sup>3</sup> CO <sub>2</sub> (4 hr) . . . . .	365	2,029	234	1,864	118	1,055
QCO <sub>2</sub> . . . . .	110	410	71	377	35	213
Inhibition (per cent) . . . . .	—	—	35	8	68	48
Reversal by neopeptone (per cent)	—	—	—	77	—	30

QCO<sub>2</sub> = mm<sup>3</sup> CO<sub>2</sub>/hour/mg *E. coli*.

## DISCUSSION

The results presented in this report show that the inhibition of carboxylase activity of *E. coli* by sulfathiazole is counteracted by neopeptone. Under similar conditions the high inhibitions exercised on *E. coli* carboxylase by *para*-, *meta*-, and *ortho*-aminobenzenesulfonamides are also counteracted by neopeptone. In contrast, the inhibition by *p*-aminobenzoic acid is not counteracted by neopeptone. These results indicate that neopeptone manifests greater affinity for carboxylase than these sulfonamides.

A human serum globulin fraction, composed of a mixture of *alpha* and *beta* globulins, exercised strong inhibitory effect on the carboxylase activity of *E. coli*. This inhibition likewise was found to be reversible by neopeptone.

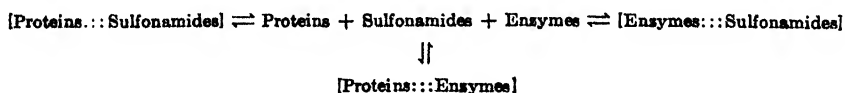
Human serum albumin also exercised a measurable degree of inhibition on *E. coli* carboxylase. In this capacity, serum albumin was found to be incapable of counteracting the inhibition exercised by *para*-aminobenzenesulfonamide and *para*-aminobenzoic acid. In contrast, the inhibitions by *meta*- and *ortho*-aminobenzenesulfonamides were abolished. With the *meta* isomer, in the presence of serum albumin, 40 per cent increase in the activity of carboxylase was observed. These facts show that the selective action of *p*-aminobenzenesulfonamide on *E. coli* is resistant to the reversing action of serum albumin, whereas the action by the other isomers is easily counteracted.

The resistance to 2 per cent human serum albumin of the inhibitory action

exercised on a respiratory enzyme by *p*-aminobenzenesulfonamide (sulfanilamide), and the ease with which serum albumin abolishes the inhibitory effects of *meta* and *ortho* isomers, appears to contribute to an explanation of the antibacterial chemotherapeutic activity of the former, and the absence of activity of the latter two, in clinical practice. It is to be noted that the sulfonamide-antagonizing property of neopeptone is only of theoretical, whereas that of serum albumin is both of theoretical and practical, interest. In this connection a study with *E. coli* by Davis (1943) is of interest. His findings and ours (Sevag, Henry, and Richardson, 1943) appear to corroborate each other. Davis found that the neutralization by 3 per cent human serum albumin of the bacteriostasis exercised by sulfonamides was most marked for sulfathiazole and least marked for sulfanilamide; with sulfapyridine and sulfadiazine it was intermediate but variable. Our results with sulfanilamide, which inhibited *E. coli* carboxylase, in the presence of 2 per cent fractionated serum albumin, appears to compare favorably with the results obtained by Davis.

In this connection reference to an earlier study is also of interest. Axmacher and Ludwig (1936) investigated the inhibitory effects of several aromatic sulfonic acids and germanin on the activity of a carboxylase preparation from brewers' bottom yeast (a clear, macerated yeast extract). In  $3 \times 10^{-3}$  M and  $3 \times 10^{-4}$  M concentrations germanin exercised, respectively, 95 and 91 per cent inhibition on carboxylase. Blood serum (final concentration in reaction system, 30 per cent) and 0.5 per cent Witte peptone were tested with respect to their antagonistic action on the inhibition by germanin. Blood serum completely reversed the inhibition by  $3 \times 10^{-4}$  M germanin. Peptone reduced the 91 per cent inhibition by  $3 \times 10^{-4}$  M germanin to 2 per cent, but exercised no antagonistic action against the 95 per cent inhibition by  $3 \times 10^{-3}$  M germanin. Evidently there is a critical concentration for each substance involved in these reactions. One-half per cent gelatin showed no reversing action in this respect.

In formulating the mechanism of these antagonistic reactions, it appears to be clear that proteins and carboxylase compete for sulfonamides (or germanin), and proteins compete with the drugs for carboxylase. According to Davis, the albumin-bound drug is ineffective, that is, the drugs enter into a "combination" with serum proteins. In view also of the fact that serum proteins inhibited carboxylase, it must be concluded that serum proteins also combine with the enzyme. Under these conditions, they may or may not exercise inhibitory effect on the activity of the enzyme, but they will be capable of preventing an inhibitory union between the drug and the enzyme. These observations can be expressed by the following scheme:



(::: indicates a reversible combination.)

That proteins enter into a combination with enzymes is evident also from the following experimental facts: inhibition of pepsin and trypsin by combining



with peptonelike substances (Northrop, 1922); irreversible combination between urease and trypsin (Tauber and Kleiner, 1931); formation of "edestin-pepsin" complex (Northrop, 1933); inhibition of milk-coagulating properties of pepsin and rennin (Tauber, 1934); reversible inactivation of tobacco mosaic virus by trypsin (Bawden and Pirie, 1937); a reversible, mutually inhibitory combination between ascarase and trypsin (Sang, 1938); and a reversible combination between *d*-ribonuclease and tobacco mosaic virus (Loring, 1942). (For a comprehensive discussion of these combinations, see Sevag, 1945.) Only a careful consideration of these factors can orient us in formulating the true mechanism of the action of sulfonamides.

#### SUMMARY

Neopeptone counteracted the strong inhibitory effects exercised on the carboxylase activity of *Escherichia coli* by *para*-, *meta*-, and *ortho*-aminobenzenesulfonamides, but showed no effect on the inhibition by *p*-aminobenzoic acid. Neopeptone exercised no inhibition on carboxylase.

Neopeptone counteracted also the inhibition exercised on the carboxylase activity of *E. coli* by human *alpha* and *beta* globulins.

Human serum albumin counteracted the inhibitory effects exercised on the carboxylase activity of *E. coli* by *meta*- and *ortho*-aminobenzenesulfonamides, but had no effect on those exercised by *p*-aminobenzenesulfonamide and *p*-aminobenzoic acid. The relation of these observations to the clinical significance of the active drugs is discussed.

A scheme is presented which shows that drugs can combine reversibly with enzyme proteins. It also shows that foreign proteins may exercise their anti-sulfonamide action by combining with either enzyme proteins or drugs.

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# PENICILLIN. III. THE STABILITY OF PENICILLIN IN AQUEOUS SOLUTION<sup>1, 2</sup>

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The instability of penicillin in aqueous solution has been known since the time of its discovery by Fleming (1929). However, beyond the general recognition that excess acidity or alkalinity caused rapid inactivation, little work has been reported on the inactivation of penicillin over a wide pH range and at different temperatures. The maximum stability range of a barium salt of penicillin tested in aqueous solution by Abraham and Chain (1942) was between pH 5.5 to 7.5. Activity was retained at 2 C for several months, at 25 C for several weeks, and at 37 C for 24 hours, whereas most of the activity was lost in 30 minutes at 100 C. Rammelkamp and Helm (1943) studied the stability of penicillin during a 24-hour period at 5 C and 37 C in veal infusion broth at pH 2, 3, 4, 5, and 7.3. Although the original concentration of penicillin was low (0.625 Oxford units per milliliter), they were able to show rapid inactivation at pH 2 and 4 at both temperatures, partial inactivation at pH 5 and 37 C, and no inactivation at pH 4 and 5 at 5 C. Foster and Wilker (1943) conducted similar experiments in buffer solutions at pH 2.0, 2.6, 2.9, 4.8, 5.8, 6.8, 7.9, 9.4, and 10.3. The initial concentration of penicillin was only 0.168 Oxford units per milliliter. They concluded that penicillin is exceedingly labile in a medium below pH 4.8 or above 7.9, losing all activity in a matter of hours. The purity of the penicillin used by Rammelkamp and Helm and by Foster and Wilker was not given, but it is apparent from the low concentration (expressed as potency) that only partially purified preparations were used.

Previous experiments conducted by the authors with crude penicillin had indicated that the optimum stability in aqueous solution was between pH 5.6 and 6.1 instead of at pH 7.0, which had been previously considered as the stability optimum. Furthermore, it was expected that crude penicillin would prove to be less stable than the crystalline material, and examination of the data presented here substantiates this supposition.

<sup>1</sup> Presented by Robert G. Benedict at the Annual Meeting of the Society of American Bacteriologists, New York, New York, May 3-5, 1944.

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<sup>4</sup> This is one of four regional laboratories operated by the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

The present investigations differ essentially from the previous studies in that a crystalline sodium salt of penicillin was employed. The initial concentration was of sufficient potency that reasonably accurate determinations of the rate of decomposition could be made. The data cover a pH range of 2.0 to 11.0 and a temperature range of 0 C to 37 C.

#### MATERIALS AND METHODS

Two samples of penicillin were employed in this work. Both were prepared from crude penicillin obtained from submerged growth of *Penicillium notatum*, NRRL No. 832. A pure crystalline preparation of the sodium salt of penicillin was used for most of the determinations. In addition, the stability of a partially purified penicillin was compared with that of the crystalline salt at pH 2.0 only.

The buffer systems employed were those whose buffering capacities were near maximum for the desired hydrogen ion concentration. The systems used were (1)  $\text{H}_2\text{SO}_4\text{-H}_3\text{PO}_4\text{-KOH}$  (approximately 0.2 N) for pH 2.0 and 3.0; (2) MacIlvaine's  $\text{C}_6\text{H}_8\text{O}_7\cdot\text{H}_2\text{O}$  (citric acid)- $\text{Na}_2\text{HPO}_4$  for pH 4.0 and 5.0; (3)  $\text{K}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$  for pH 5.5, 5.8, 6.0, 6.5, 7.0, and 7.5; (4)  $\text{H}_3\text{BO}_3\text{-NaOH}$  plus  $\text{HCl}$  for pH 8.0 and 9.0; and (5)  $\text{H}_3\text{BO}_3\text{-NaOH}$  plus  $\text{NaOH}$  for pH 10.0 and 11.0. Each buffer was carefully checked with a Beckman pH meter, and small amounts of chloroform and toluene were added to prevent growth of microorganisms.

Temperatures used in these investigations were 0, 10, 15, 24, 30, and 37 C. Constant temperature baths were employed for 24, 30, and 37 C. A well-insulated cold water bath was maintained at 15 C for tests at pH 2.0. For long-term experiments at 10 C, a bath was installed in a refrigerator maintained at that temperature. A large Dewar flask, filled with cracked ice and stoppered with cork, provided an excellent bath for 0 C studies.

For each experiment with the crystalline sodium salt of penicillin, a carefully weighed sample in a volumetric flask was dissolved in buffer at the desired temperature to give a final concentration of 100 to 125 Oxford units per milliliter. The partially purified penicillin solution was transferred by pipette and diluted to approximately the same concentration. After taking a zero time sample, the penicillin buffer mixture was immersed in a bath held at the required temperature. Additional samples were taken at intervals throughout the experiment. Each sample withdrawn was immediately diluted with ice-cold phosphate buffer at pH 6.0 to stop penicillin inactivation and to provide suitable dilutions for assay. These were stoppered and refrigerated at 4 C until assayed in the afternoon or evening of the same day. The cylinder-plate method of Schmidt and Moyer (1944) was utilized for all assays, and the values from two or three plates were averaged to determine each point. Final pH values were determined on the penicillin buffer mixtures at the conclusion of each experiment.

#### EXPERIMENTAL RESULTS

The first inactivation studies were made at pH 2.0 and 0 C. A trial run was conducted to determine the approximate rate of inactivation and time

intervals for taking samples. After repeating the tests at pH 2.0 and 0 C, tests were conducted at 10 C and 24 C with approximately 24 samples taken

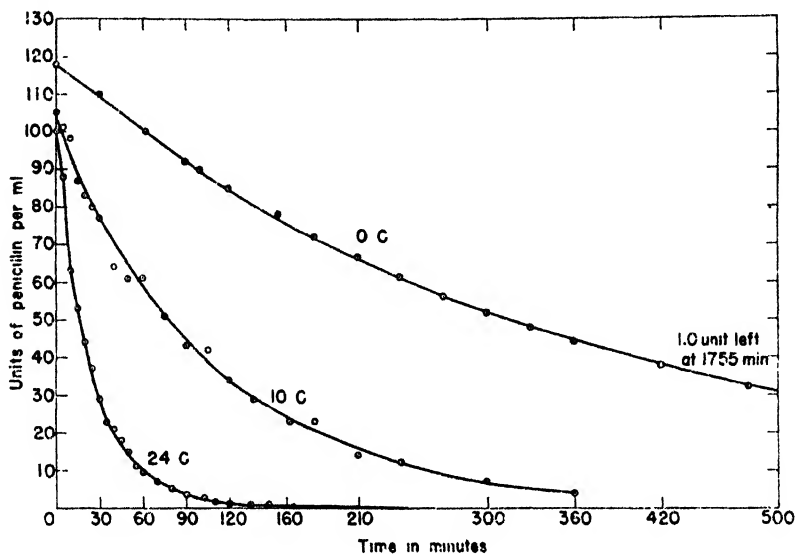


FIG. 1. DECOMPOSITION OF CRYSTALLINE PENICILLIN AT pH 2.0

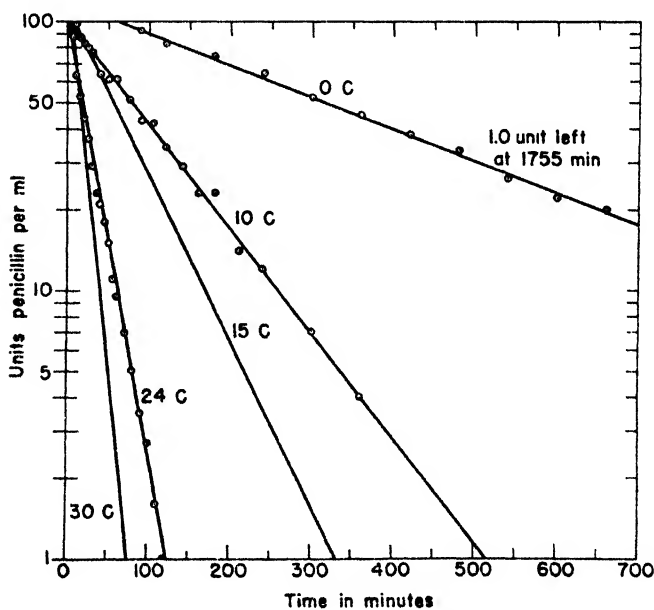


FIG. 2. EFFECT OF VARIOUS TEMPERATURES ON THE DECOMPOSITION OF CRYSTALLINE PENICILLIN AT pH 2.0

during each run. When the penicillin concentration is plotted against time, logarithmic curves are obtained (figure 1). If semilogarithmic paper is used, the points all fall on a straight line (within experimental error), as shown (figure

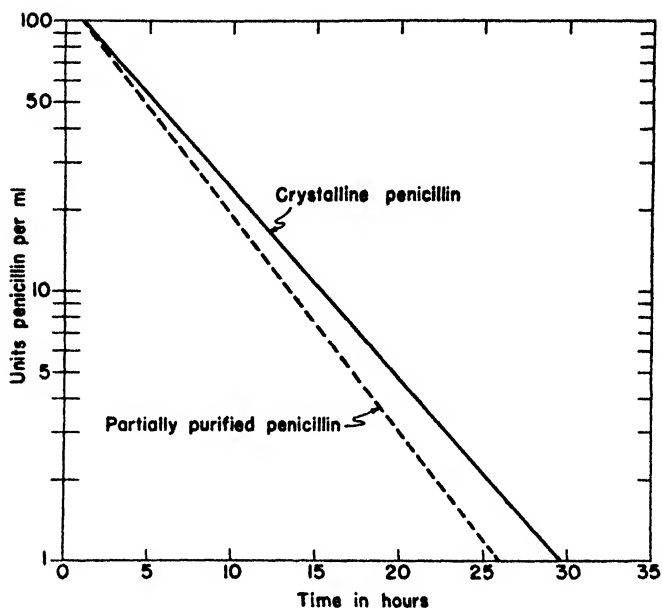


FIG. 3. DECOMPOSITION OF CRYSTALLINE AND PARTIALLY PURIFIED PENICILLINS AT pH 2.0-0°C

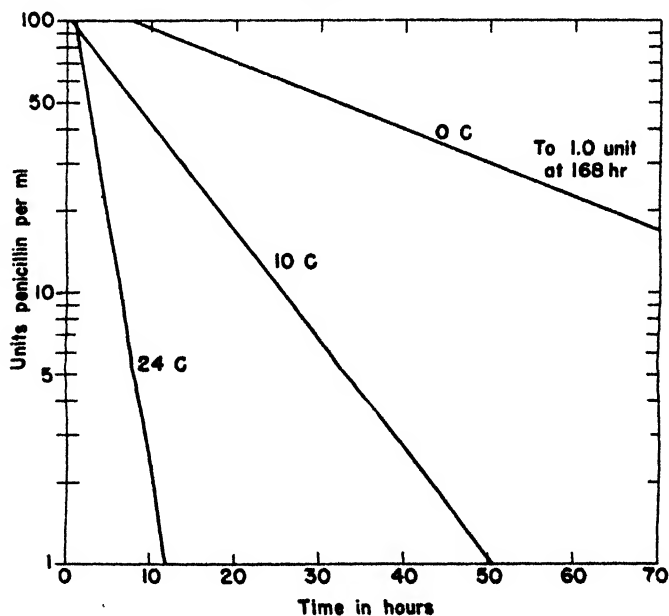


FIG. 4. DECOMPOSITION OF CRYSTALLINE PENICILLIN AT pH 3.0

2). The distribution of points for the slopes shown in this figure are considered representative of those obtained throughout the work. The effect of temperature is such that the times required to destroy 50 per cent of the penicillin at pH

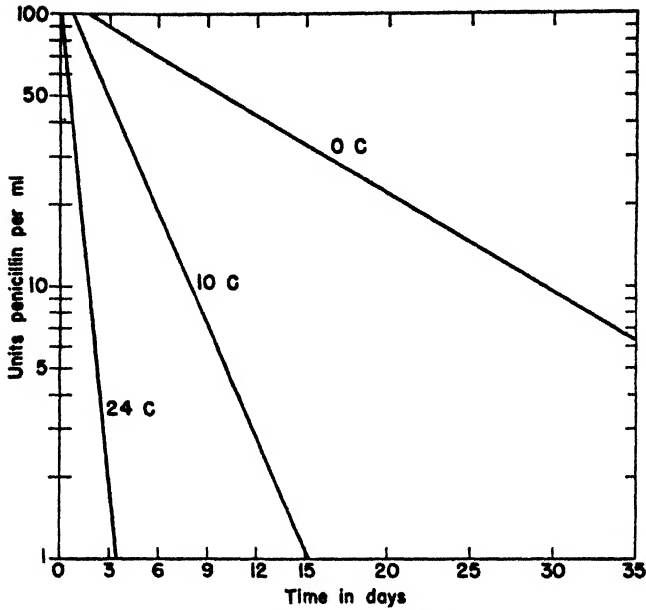


FIG. 5. EFFECT OF VARIOUS TEMPERATURES ON CRYSTALLINE PENICILLIN AT pH 4.0

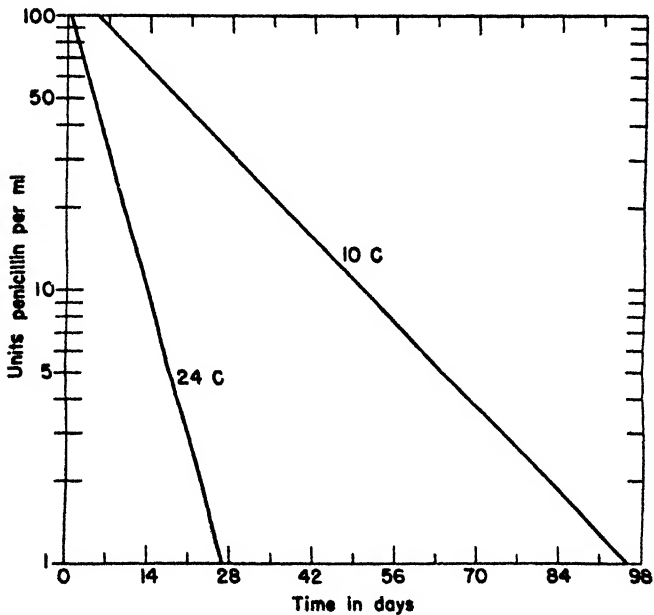


FIG. 6. STABILITY OF CRYSTALLINE PENICILLIN AT pH 5.0

2.0 are approximately 5 hours at 0 C, 77 minutes at 10 C, and 17 minutes at 24 C. Tests were run at 15 and 30 C, and the slopes for these temperatures are also shown in figure 2. The points used to determine them were purposely

omitted to avoid confusion. The additional information enabled one of us (Oleson) to derive certain mathematical formulae pertaining to the inactivation of crystalline penicillin at pH 2.0. This material may be found in a separate section of this paper.

A comparison of the stability of partially purified penicillin with that of the crystalline material was made at pH 2.0 and 0 C. Figure 3 shows the results of this comparison. The increased rate of destruction in the impure material is

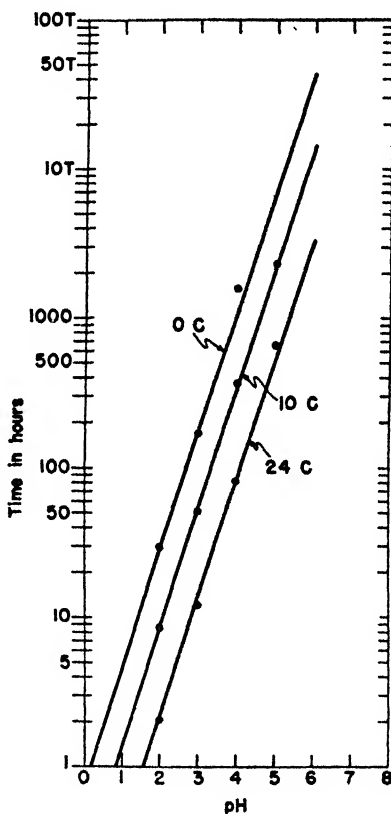


FIG. 7. TIME IN HOURS REQUIRED TO DESTROY 99 PER CENT OF CRYSTALLINE PENICILLIN AT 0, 10, AND 24 C PLOTTED AGAINST pH

probably due to the presence of certain impurities which react with penicillin, the curve representing the summation of this effect and that resulting from the acidity of the solution.

The stability of the crystalline salt was tested at pH 3.0, 4.0, and 5.0 using 0, 10, and 24 C as the temperatures. For each unit rise in pH, the stability increased so markedly that the interval between taking of samples in the pH 5.0 series was increased to one day. Figures 4 and 5 show the slope of the lines for pH 3.0 and 4.0, respectively. After sampling at intervals for 25 days from the pH 5.0 and 0 C flask, the penicillin concentration was still above 100 units

per milliliter. This experiment was discontinued for that reason and accounts for the absence of a pH 5.0 and 0 C slope in figure 6. Some rather interesting data were obtained by plotting pH (on semilogarithmic, 5-cycle paper) against the time needed to destroy 99 per cent of the penicillin at 0, 10, and 24 C (figure 7). The slopes thus attained are parallel and give some indication of the stability at pH 6.0. On the basis of this information, it was not practical to make runs with pH 6.0 and temperatures of 0 and 10 C because the predicted times for 99 per cent inactivation were 42,000 and 14,500 hours, respectively.

*Summary of mathematical data pertaining to decomposition of penicillin at pH 2.0*

Previous reference has been made in this paper to the derivation of certain mathematical formulae by one of the authors. The data presented at this time are limited to a summary of this work since the mathematical calculations involve considerable detail.

The decomposition of penicillin at a constant pH of 2.0 appears to be a first-order irreversible reaction. The results may be expressed as follows:

$$\log_{10} \frac{C_0}{C} = K^1(\theta - \theta_0) \quad (1)$$

$C_0$  = concentration of penicillin at time  $\theta_0$ .

$C$  = concentration of penicillin at time  $\theta$ .

$K^1$  = reaction rate constant.

$C_0$  and  $C$  may be expressed in any units.

$\theta$  is expressed as minutes.

$K^1$  has the dimension,  $\text{min.}^{-1}$

The experimentally determined constant  $K^1$  was obtained at pH 2.0 at 0, 10, 15, 24, and 30 C. The reaction rate constant was correlated with the Arrhenius equation to secure an equation with  $K^1$  as a function of temperature. The result is equation (2).

At pH = 2.0

$$\log_{10} K^1 = -3818 T + 11.05874 \quad (2)$$

$T$  = temperature of reaction, °K ( $273 + t$ , °C.).

Equation (2) may be used to calculate  $K^1$  for any temperature ( $-10$  C to  $+40$  C) at pH = 2.0. The determined value of  $K^1$  is then used in equation (1) to determine the extent of decomposition at any time, if the original concentration and time at which the reaction begins is known.

The rate of decomposition may be determined by calculating  $K^1$  using (2) and substituting in the equation

$$\frac{dC}{d\theta} = -2.3026 K^1 C \quad (3)$$

where  $C$  is expressed as desired. In the work done it would be (units per milliliter) and since  $K^1$  is ( $\text{min.}^{-1}$ ) then the rate is expressed dimensionally as units of penicillin per milliliter disappearing per minute.



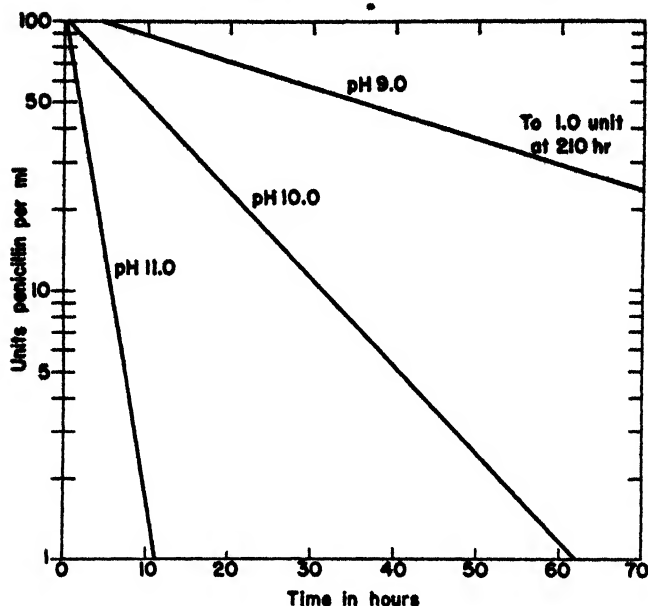


FIG. 8. STABILITY OF CRYSTALLINE PENICILLIN AT pH 9.0, 10.0, AND 11.0 (24 C)

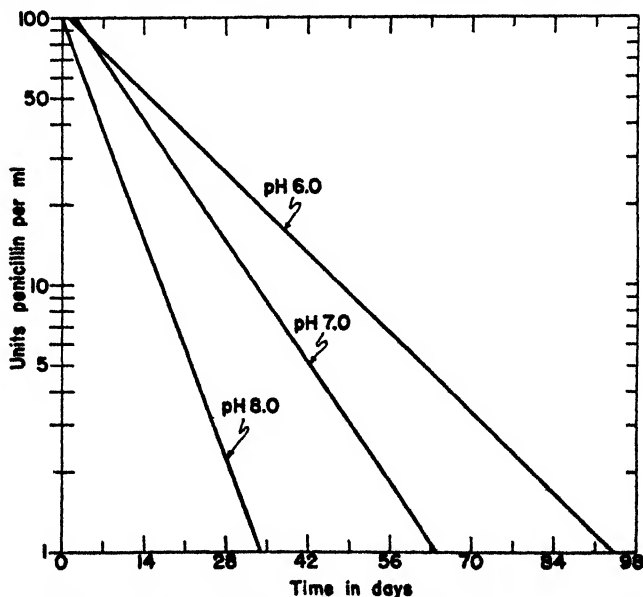


FIG. 9. STABILITY OF CRYSTALLINE PENICILLIN AT pH 6.0, 7.0, AND 8.0 (24 C)

*Inactivation of crystalline penicillin at pH 6.0 to 11.0*

Detailed investigations of the same nature as those from pH 2.0 to 5.0 were not possible in this phase of the work, due to the large numbers of assays necessary for other penicillin projects in the laboratory. The inactivation of penicillin at pH 9.0, 10.0, and 11.0 (24 C) is shown in figure 8.

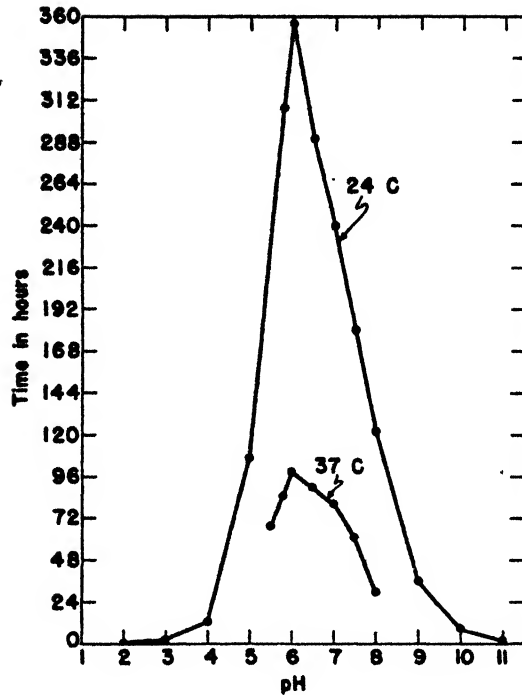


FIG. 10. EFFECT OF pH ON TIME REQUIRED TO DECOMPOSE 50 PER CENT OF CRYSTALLINE PENICILLIN IN AQUEOUS SOLUTION AT 24 AND 37 C

TABLE 1

*The effect of pH and temperature on the half-life of penicillin  
(time in hours to inactivate 50 per cent)*

pH	0 C	10 C	24 C	37 C
2.0	5.2	1.28	0.283	—
3.0	32.52	8.2	2.33	—
4.0	259.2	68.4	13.2	—
5.0	2,000*	458.4	107.2	—
5.5	—	—	—	67.2
5.8	—	—	318	85.2
6.0	—	—	356	99
6.5	—	—	290.5	90.0
7.0	—	—	252	81.6
7.5	—	—	180	61.2
8.0	—	—	122.4	29.8
9.0	—	—	36.0	—
10.0	—	—	9.7	—
11.0	—	—	1.7	—

\* Estimated.

Stability tests were run at pH 6.0, 6.5, 7.0, 7.5, and 8.0 at 24 C. Samples were taken for dilution at 1-day intervals and 16 points were used to determine the slope of each line. Three of these slopes (for pH 6.0, 7.0, and 8.0) are shown

in figure 9. From these results it appeared quite certain that pH 6.0 was the optimum stability point for crystalline penicillin in aqueous solution. Before a stability curve at 24 C was drawn, tests were conducted with pH 5.5 and 5.8 at 24 C, and with pH 5.5, 5.8, 6.0, 6.5, 7.0, 7.5, and 8.0 at 37 C. Figure 10 shows the effect of pH on the time required to inactivate 50 per cent of a solution of crystalline penicillin at 24 C. The data available from the 37 C investigations (pH 5.5 to 8.0) are also included. A more complete summary of the effect of temperature and pH on the "half-life" of penicillin (time required to inactivate 50 per cent) is shown in table 1. These data show the rapid increase in stability of penicillin as the hydrogen ion concentration decreases (pH 2.0 to 6.0) with subsequent increase in decomposition as the concentration of hydroxyl ions becomes greater.

#### DISCUSSION

The authors hope that this work will greatly assist in the clarification of previous knowledge concerning the instability of penicillin. The program of work was originally outlined to include decomposition studies at pH 2.0 to 5.0 only. Consequently, more data were gathered concerning the stability in that pH range than at higher pH values. Reasonably accurate results were more easily attained when an inactivation experiment was completed in 1 to 5 days or less, since slight variations in pH or temperature were then less likely to occur. These sources of error were much harder to control in long-term experiments. Destruction of penicillin in the runs with pH 4.0 at 0 C and pH 5.0 at 10 C was so slow that accurate slopes were difficult to obtain within the time allowed for the experiments. Any microbiological method of assay is subject to certain errors, regardless of the competency of the investigator employing it. The cylinder-plate assay method of Schmidt and Moyer (1944), used in this work, may be in error 15 to 20 per cent. However, the writers, and the authors of the paper cited, are of the opinion that in the experiments reported here  $\pm 8.0$  per cent is a more likely error than 15 to 20 per cent. The assay errors in these investigations were greatly reduced by using two or three plates for each diluted sample so that 6 to 9 values were averaged for each point on a slope or curve. It is believed that the inactivation caused by the buffers in these experiments was brought about by excess hydrogen or hydroxyl ions and not by the presence of other ions in the systems. It is possible that different results might have been obtained with other buffers, but this phase was not investigated.

#### SUMMARY

A series of graphs has been presented to show the stability of a crystalline sodium salt of penicillin in aqueous solution at pH 2.0 to 11.0 at various temperatures. When compared with a solution of the crystalline salt at pH 2.0 and 0 C, a partially purified solution of penicillin under the same conditions was found to be less stable.

It appears that the destruction of penicillin in aqueous solution is a first-order irreversible reaction.

The time required to destroy a solution of crystalline penicillin at pH 2.0 between  $-10^{\circ}\text{C}$  and  $+40^{\circ}\text{C}$ , and the rate of destruction at any chosen point during that time, may be calculated by the use of formulae derived from the data presented in this paper.

#### ACKNOWLEDGMENT

We are indebted to Dr. Frank Stodola and coworkers of the Fermentation Division who kindly furnished the samples of penicillin employed in this work.

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# THE GLANDERS ORGANISM WITH REFERENCE TO ITS CELL INCLUSIONS

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Each of a number of current texts in bacteriology refers to the irregular staining of the glanders organism, although no satisfactory explanation is offered for this feature of *Malleomyces mallei*. With reference to this subject Zinsser and Bayne-Jones (1939) state: "Stained in the usual manner with methylene blue, . . . [the glanders organism] shows marked irregularity in staining qualities; granular deeply staining areas alternating with very faintly stained or entirely unstained portions. This diagnostically helpful characteristic has been variously interpreted as a mark of degeneration or a preparatory stage for sporulation." These authors conjecture that the irregular staining is probably due to neither of the two explanations but rather to "an inherent irregularity in the normal protoplasmic composition of the bacillus, not unlike that of *B. diphtheriae*." Likewise Jordan and Burrows (1941) state: "Thought by some to resemble spores, granular material within the cells is probably volutin or similar substances." Similar references to the irregular staining of the glanders organism are made by Topley and Wilson (1936), by Belding and Marston (1938), by Bergey (1939), and by Fairbrother (1941), without an attempt to account for such irregularity.

Since we felt that the irregular staining of the glanders organism might be attributed to certain cell inclusions, we decided to study this feature of the organism with particular interest in determining whether or not fat bodies are present.

## MATERIALS AND METHODS

The following strains of *Malleomyces mallei* were obtained:

<i>M. mallei</i> 2024. . . . .	The University of Michigan
<i>M. mallei</i> F. . . . .	U. S. Department of Agriculture
<i>M. mallei</i> 2024 . . . . .	U. S. Department of Agriculture
<i>M. mallei</i> 8309 . . . . .	American Type Culture Collection
<i>M. mallei</i> 8310. . . . .	American Type Culture Collection
<i>M. mallei</i> 77. . . . .	American Type Culture Collection

Organisms from plain agar and glycerol glucose agar cultures were stained with gentian violet, methylene blue and acid fuchsin. In addition, organisms from slants of plain agar, tryptose agar, and glycerol glucose agar were obtained for Sudan black B wet mounts prepared in accordance with Hartman's procedure (1940). To obtain a wet mount preparation of this pathogen which could

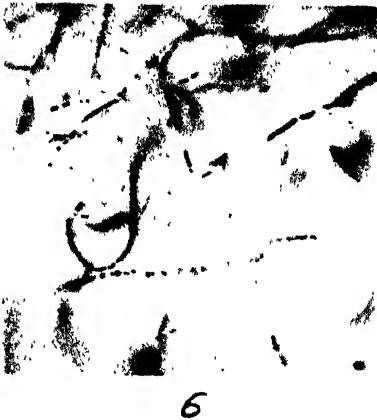


FIG. 1. ACID FUCHSIN STAIN OF 3-DAY-OLD *MALLEOMYCES MALLEI* F (GROWN ON GLYCEROL GLUCOSE AGAR)

FIG. 2. GENTIAN VIOLET STAIN OF 3-DAY-OLD *MALLEOMYCES MALLEI* 2021 (MICHIGAN) GROWN ON GLYCEROL GLUCOSE AGAR

FIG. 3. GENTIAN VIOLET STAIN OF 3-DAY-OLD *MALLEOMYCES MALLEI* 2021 (MICHIGAN) GROWN ON GLYCEROL GLUCOSE AGAR AND SHOWING FILAMENTOUS FORMS

FIG. 4. SUDAN BLACK B WET MOUNT PREPARATION OF 3-DAY-OLD TRYPTOSE AGAR CULTURE OF *MALLEOMYCES MALLEI* 2024 (MICHIGAN)

FIG. 5 AND 6. SUDAN BLACK B WET MOUNT PREPARATION OF 3-DAY-OLD TRYPTOSE AGAR CULTURE OF *MALLEOMYCES MALLEI* 2024 (MICHIGAN) SHOWING FILAMENTOUS FORMS

be studied with reasonable safety, we used large slides (2" by 3") with cover slips placed in the center and sealed with petrolatum. Similarly, organisms were subjected to Scharlach R and to Eisenberg's iodine-fuchsin method of staining fat (1909).

Cultures grown on tryptose agar were stained with methylene blue in an attempt to determine whether or not volutin is deposited.

#### RESULTS

Figure 1 illustrates the irregular staining of glanders cells with acid fuchsin. Certain of the cells show unstained oval areas with the surrounding portion stained. Figure 2 presents a similar picture with cells stained irregularly by gentian violet, whereas figure 3 illustrates the long filamentous forms.

Figures 4, 5, and 6 show the glanders organism in a Sudan black B wet mount. The round-stained granules, considered to be fat bodies, show up rather prominently both in the filamentous forms and in the rodlike forms.

With Eisenberg's iodine-fuchsin method of staining lipid bodies, the granules are also stained. With Scharlach R the results are not so clear; however, this procedure for staining bacterial fat in general has not been so reliable in the authors' hands as the first two procedures.

Each strain was found to deposit fat bodies. Moreover, such fat bodies were found in organisms grown on plain agar, tryptose agar, and glycerol glucose agar.

With cultures grown on tryptose agar and stained with Loeffler's methylene blue, volutin bodies could not be demonstrated. Moreover, 1 per cent sulfuric acid completely decolorized the cells, whereas control procedures, using a *Corynebacterium diphtheriae* culture grown on Loeffler's coagulated serum, revealed the presence of an abundant amount of volutin.

#### SUMMARY

The irregular staining of the glanders organism, referred to frequently in the literature, is considered to be due in a large part, if not entirely, to the presence within the protoplasm of lipoidal granules which fail to stain readily with the usual aniline dyes. Such granules do, however, stain readily with Sudan black B or with the iodine-fuchsin procedure. They are deposited in cells whether grown on plain agar, tryptose agar, or glycerol glucose agar.

Attempts to demonstrate the presence of volutin were unsuccessful.

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# IMPROVEMENTS IN THE CYLINDER-PLATE METHOD FOR PENICILLIN ASSAY<sup>1</sup>

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## INTRODUCTION

The most widely used technique for penicillin assay is the so-called cylinder-plate method of Abraham *et al.* (1941), with various modifications such as those described by Foster and Woodruff (1944) and by Schmidt and Moyer (1944). In using this method for large numbers of assays we have found it advantageous from the standpoint of time involved and reproducibility of the method to use a  $4\frac{1}{2} \times 11\frac{1}{2}$  inch, rectangular, culture plate on which 40 cylinders can be placed.

As compared with conventional petri plates, these culture plates increase efficiency in two ways, namely, by decreasing the proportion of cylinders devoted to standards and by reducing the time required to pour, flood, and wash the plates needed for a given number of assays. In addition to increased efficiency, increased reproducibility is obtained by using culture plates with flat bottoms and pouring them on a carefully leveled surface. This insures a degree of uniformity of depth of culture medium not attainable in petri dishes because of irregularities in their bottoms. Such irregularities are of sufficient magnitude to constitute a real source of error.

## EQUIPMENT

Culture dishes are made of stainless steel rims placed on 6 x 13 inch sheets of single strength window glass. A second sheet of glass of like dimensions is used as a lid. The rims are made of 24-gauge, stainless steel strips bent to form  $\frac{1}{4} \times \frac{3}{4}$  inch angles. From these angle strips rectangular rims  $\frac{3}{4}$  inches high,  $4\frac{1}{2}$  inches wide, and  $11\frac{1}{2}$  inches long are made with the  $\frac{1}{4}$ -inch flange extending outward from the bottom edge. Such rims are illustrated in figures 1 and 2. They are attached at their ends to the bottom plate with 1-inch, gummed paper tape strips. Leakage of the medium under the rim is prevented by running 2 medicine droppers of liquid agar medium under the rim and allowing this to set as a seal. Plates are poured on leveled plate glass sheets.

To facilitate placing cylinders on inoculated plates a leucite guide, as illustrated in figure 2, is used. This is constructed of two sheets of  $\frac{3}{8}$ -inch leucite or similar plastic bolted together in parallel fashion in such a way as to allow a stainless steel slide to move freely between them and be removed from one side. Through the two sheets of leucite four rows of ten holes are drilled one inch apart each way to act as guides for the cylinders. These holes must be properly aligned

<sup>1</sup> The method reported in this paper was developed in connection with work done under the Office of Production Research and Development, War Production Board, Contract 169.

and of such size as to guide the cylinders accurately while allowing them to fall freely as the slide is removed. The holes in the lower plate are slightly enlarged on the top side with a taper reamer. Those on the top plate are beveled with a countersink to facilitate placing cylinders in them.

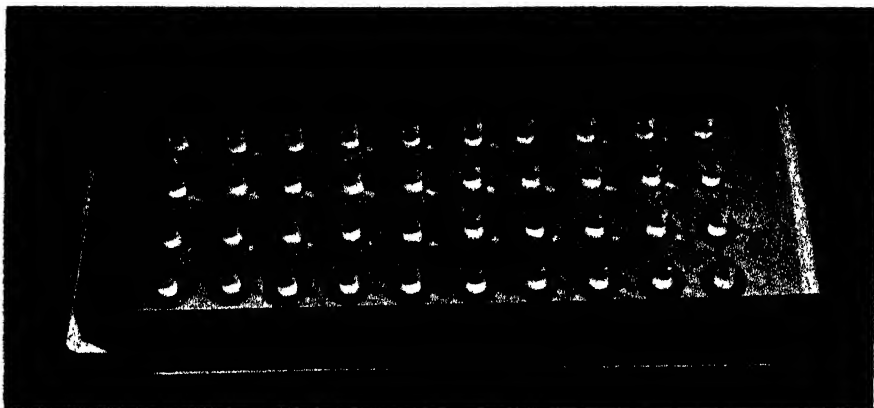


FIG. 1. VIEW OF CULTURE PLATE SHOWING ARRANGEMENT OF CYLINDERS

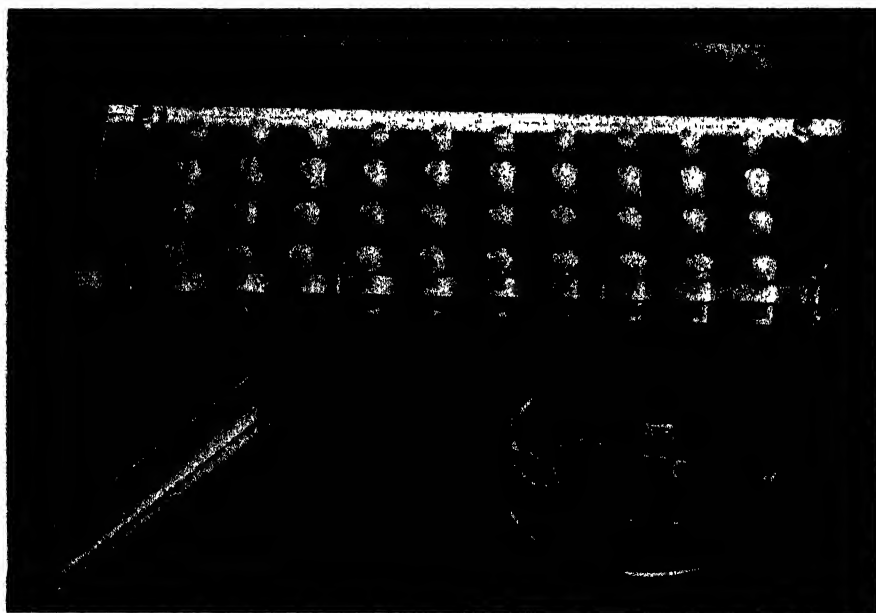


FIG. 2. VIEW SHOWING LEUCITE GUIDE, CYLINDERS, AND METAL RIM

Guides are used by loading the top plate with cylinders, placing the guide over the culture plate, and allowing the cylinders to drop into place by removing the slide. If one end of the guide is held in place on the plate while the other is lifted, cylinders are not moved on removal of the guide. Guides are cleaned daily with 70 per cent alcohol and stored in a dust-free cabinet.

## CYLINDER-PLATE METHOD FOR PENICILLIN ASSAY

Cylinders are made of standard pyrex tubing selected for an outside diameter of  $7.9 \pm 0.1$  mm and cut into sections one cm long. The ends are ground flat on a carborundum wheel and then fire-polished just enough to produce a glaze but not enough to round the cylinder edges.

### ASSAY PROCEDURE

With respect to test organisms, media, buffer, dilutions, time, and temperature of incubation, etc., we follow the recommendations of Schmidt and Moyer as given in their 1944 paper and as subsequently modified by them. We recommend that investigators making extensive use of penicillin assay methods communicate with the United States Department of Agriculture, Northern Regional Research Laboratory, Peoria, Illinois, Dr. R. O. Coghill, for the current recommendations of the Peoria group.

In a further effort to increase reproducibility, we attempt to keep time intervals constant between successive steps in the assay procedure. Plates are poured (83 ml per plate), allowed to set, and stored in an incubator at 44 C° for 3 to 4 hours. Using a rapidly draining pipette, the plates are flooded with 17 ml of inoculated medium. The agar is then allowed to solidify and the cylinders dropped into place. Following an interval of 15 minutes, test solutions are pipetted into the cylinders, and the plates placed in a 37 C° incubator over night (ca. 16 hours).

Inhibition zones are measured by means of a light box containing a daylight fluorescent tube under two glass plates. Between these plates is placed a celluloid sheet on which circles differing in diameter by one millimeter increments are drawn. In routine assays diameters are read to the nearest half millimeter.

On the completion of readings, the plates are flooded with lysol and subsequently dismantled for cleaning. After this they are reassembled, wrapped in paper, and sterilized by autoclaving. They are dried by exhausting the autoclave and then allowing it to develop a vacuum for 15 or 20 minutes.

### ERROR VARIANCE

To obtain an indication of the magnitude of unavoidable errors of the method an analysis of variance was made on a series of replicated runs made at levels near 1.0 unit penicillin per ml with inhibition zones read to the nearest 0.25 mm. In eight different determinations, each based on from six to nine individual tests, 75 measurements were made. The "within-series" variance was 0.1 mm in zone diameter. This corresponds to a standard error of about 0.3 mm. At a level of one unit per ml this represents approximately 0.07 unit penicillin per mm, or 7 per cent. Expressed in another way, at a level of 1 unit, about two-thirds of the individual determinations would fall within 0.07 unit of the true value. Duplicates and higher replicates would, of course, reduce the standard error of a determination inversely as the square of the number of cylinders used per determination.

The above measurement of variance does not include errors of dilution or those due to variations in standards. Neither does it include interplate varia-

tion. In our experience the plate-to-plate variation is less than that encountered with the use of petri plates.

#### SUMMARY

A  $4\frac{1}{2} \times 11\frac{1}{2}$  inch rectangular culture plate for use in the cylinder-plate method is described. A guide is used for dropping cylinders upon the inoculated culture plate. The use of this equipment saves time in large-scale assay work and permits a higher degree of standardization than does the conventional petri plate technique.

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## NOTES

### OXIDATION OF BUTYRIC ACID BY STREPTOCOCCI

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A number of cultures of the *Streptococcus mitis* group were found to oxidize butyric acid under aerobic conditions with the accumulation of hydrogen peroxide. Methylene blue would not serve as a hydrogen acceptor for this butyric acid oxidase system under the conditions tested.

The quantity of peroxide produced by these organisms, when grown in a medium containing butyrate, in some cases compared closely to that produced by pneumococci from meat infusion. In cell suspensions of these streptococci, under highly aerobic conditions, as much as 0.01 M peroxide accumulated from 0.011 M butyrate within one hour at 25 C, pH 7.2. Of all the strains tested, essentially the same qualitative results were obtained with cell suspensions and with growing cultures in a butyrate medium.

Among the 39 *Streptococcus mitis* cultures, freshly isolated from the throats of 18 persons, a relationship was noted between the ability to oxidize butyrate and other physiological characters. For example, of the 28 which failed to hydrolyze arginine, 24 oxidized butyrate; of the 11 arginine-positive strains, only 2 oxidized butyrate. This is in accord with a previously noted relationship between the ability to hydrolyze arginine and certain other physiological reactions (Sherman, Niven, and Smiley: J. Bact., 45, 249) that indicates that the *Streptococcus mitis* group may consist of more than one species.

A number of representative cultures of all of the well-known serological groups and species of streptococci were tested for the ability to oxidize butyrate, in growing culture and in cell suspension. Aside from *Streptococcus mitis*, no culture was found which possessed this property with the exception of members of the Lancefield group F and the "minute" variety of group G. All of the 5 strains of group F and 8 strains of group G, "minute" variety, definitely oxidized butyrate, but the degree of peroxide accumulation by these cultures was less than that of the *Streptococcus mitis* strains. The "nonminute" group G cultures (10 strains) were unable to attack this substance. None of the 11 pneumococcus cultures tested, representing as many serological types, were able to oxidize butyrate.

#### SUMMARY

Butyric acid is oxidized by streptococci of group F, the "minute" variety of group G, and by many strains of *Streptococcus mitis*. Other species of streptococci and the pneumococci do not appear to have this property.

## USE OF HORSE MEAT INFUSIONS IN CULTURE MEDIUMS

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Horse meat infusions have been used in Europe for many years as a basis for culture mediums. In Seattle horse meat was unrationed, plentiful, and cheap, so it was substituted in our laboratory for beef and pork in mediums used for routine classwork.

The horse meat infusion has a yellow color and pungent odor, and often a thin layer of fat appears on the surface. Glucose in the horse broth medium was found to be 0.23 g per 100 ml, whereas that in the beef broth controls was 0.08 g per 100 ml. In total N horse meat is comparable to beef and pork; but it is much higher in glycogen than either pork or beef. It is a good source of sulphur, phosphorus, and potassium, but a poor source of calcium and magnesium.

Twenty pathogenic organisms were cultured on 6 kinds of solid mediums and 11 types of liquid mediums in which horse meat infusion had been substituted for beef or pork. All cultures were controlled by beef extract and beef infusion mediums. The acid shifts were read with the Leeds Northrup potentiometer, after 4 days of incubation.

All cultures used grew well on the horse mediums. Pigment formation in *Staphylococcus aureus* was not enhanced by the use of the medium, but there was some indication that it utilized some of the sugar in the horse infusion broth.

*Streptococcus pyogenes* formed a wide zone of beta hemolysis in 18 hours on the horse infusion blood agar, whereas it appeared only in a narrow zone after 36 hours on the blood beef infusion medium. With streptococci viridans and the pneumococci studied, the same was found true in the production of alpha hemolysis.

Horse infusion was substituted for pork infusion in making the basic agar for tellurite medium. This modified medium was used with good results both in studying the characteristics of stock strains of *Corynebacterium diphtheriae* and for isolating unknown cultures. The reactions were clearer and more rapid and the colonies were larger than those found on the pork tellurite medium.

The digestion of deep meat, and of meat and egg, in tubes by the species of *Clostridium* studied was similar whether horse or beef muscle was used. However, a striking difference between their reactions on horse liver medium and beef liver medium was noted. *Clostridium tetani* was the only species which showed blackening in deep tubes of beef liver. All six strains used showed blackening in the horse liver. *Clostridium putrificum*, *Clostridium welchii* and *Clostridium botulinum* produced gas and blackening throughout the tubes. *Clostridium histolyticum* showed blackening on the surface of the medium and along the stab. In *Clostridium sporogenes* the medium was blackened only on the surface, whereas in *Clostridium tetani* the blackening was found only along the stab.

Horse meat infusion can be substituted in routine laboratory mediums and is especially useful in demonstrating hemolysis.

# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## THE EASTERN MISSOURI BRANCH

SAINT LOUIS, MISSOURI

OCTOBER 10, 1944

**TRANSMISSIBLE VARIATION OF PLAQUE-TYPE IN BACTERIOPHAGE.** *A. D. Hershey and J. Bronfenbrenner*, Washington University School of Medicine.

When plated in very soft agar (about 0.6 per cent), coliphage-PC forms plaques consisting of an area of complete lysis 0.5 mm in diameter, surrounded by a zone of partial lysis about one mm in diameter. In about 1 per cent of the plaques, a secondary zone of complete lysis appears within the halo. This may take the form of a barely visible spot or, more typically, of a radial streak or sector. Phage propagated from the sector regularly yields a variant phage characterized by larger plaques (about 2 mm in diameter), which may be nearly clear throughout, but more often gives the effect of a plaque of the parent type surrounded by a third zone of complete lysis. Propagation from normal-appearing regions of the parent plaque invariably yields phage of the parent type, which in turn gives rise to a small proportion of sectoried plaques.

In platings on 1 per cent agar the variation cannot be seen, owing to the absence of halo, although the isolated phage of variant type can be distinguished by the larger size of plaques. When a mixture of the two phages is plated, a well-marked interference between adjacent plaques of the two types may be seen, taking the form of suppression of lysis in the area receiving phage of both types.

**MAL DEL PINTO.** *R. B. H. Gradwohl*, Gradwohl Laboratories, St. Louis, Missouri.

Mal del Pinto is a disease which is prevalent in the State of Guerrero, Mexico. It is also found in Colombia, Venezuela, Peru, and some of the Central American countries and West Indian islands. It is transmitted by direct contact and can be reproduced by direct inoculation.

The specific organism, *Treponema cara-*

*teum*, was discovered by Leon y Blanco and affirmed by Brumpt. The organism is found in the skin and can easily be demonstrated by pinching the areas affected with a pair of forceps and making a scraping. Examination is then accomplished by dark field illumination of the lymph.

The epidermis shows hyperkeratosis, acanthosis, intercellular edema, and lymphocytic infiltration. Later on, there is deep pigmentation, and in the vitiliginous areas there is distinct atrophy of the epidermis with flattening or loss of the rete pegs and complete disappearance of pigment in both epidermis and cutis. There is a primary lesion, a secondary period of generalized eruption, and a late dyschromic stage. The types commonly seen of Mal del Pinto are blue and white pinto. The so-called red cases look like a flushing of the skin.

**SUCCINCHLORIMIDE AS A WATER DECONTAMINANT.** *G. F. Reddish*, Lambert Pharmaceutical Company, St. Louis, Missouri.

Succinchlorimide has been put into a tablet containing a dispersing agent. When added to water it disperses within 10 to 15 seconds and completely dissolves within from 1.5 to 2 minutes at 76 F.

When a 2-grain tablet containing 12 mg of succinchlorimide is added to a liter of tap water containing 68,000 *Shigella dysenteriae* and 304,000 *Eberthella typhosa* per ml, all of these organisms are killed within 5 to 10 minutes at 73 F. When such a tablet is added to a liter of raw river water containing 27,000 *S. dysenteriae* and 75,000 *E. typhosa* per ml, all are killed within 5 to 10 minutes at 36 F. When the same dosage of succinchlorimide is added to a liter of swamp water (15 ppm chlorine demand) containing 800,000 *S. dysenteriae* and 288,000 *E. typhosa* per ml, all are killed within 5 to 10 minutes at 80 F.



In order to kill dysentery amoeba cysts, five times this dosage is required. At this concentration the cysts of *Endamoeba histolytica* are killed within 20 minutes.

**PHYSIOLOGICAL PROPERTIES OF ADAPTIVE ENZYME FORMATION.** *S. Spiegelman*, Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis, Mo.

Two diploid strains of *Saccharomyces cerevisiae* were employed in studying the physiology of the adaptation to galactose fermentation. All the experiments were performed on stationary, nondividing suspensions in  $M/15\text{ KH}_2\text{PO}_4$  to which galactose was added.

With the Warburg manometric technique it was found that the adaptation time (time required to reach a  $Q_{\text{CO}_2}^N$  value of 100) increased as the endogenous carbohydrate reserves were decreased by dissimilation in

the absence of external substrate. Nevertheless, the galactozymase was found to form even after all the oxidizable reserves were exhausted as indicated by negligible  $Q_{\text{O}_2}$  values.

A more detailed examination of the course of the adaptation indicated that oxidation of the galactose provided the metabolic energy necessary for the cells to adapt to its fermentative utilization. It was found that cells unable to utilize the galactose anaerobically, nevertheless possessed a mechanism for its aerobic oxidation. Thus adaptation was not to galactose utilization per se, but involved only the synthesis of the enzymes required for its fermentation. It was further found that the preadaptive oxidation of galactose apparently was mediated through the same mechanism used in the endogenous metabolism. A competitive interaction could be established between the endogenous substrate and the galactose in the preadaptive period.

## VIRGINIA BRANCH

JEFFERSON HOTEL, RICHMOND, VIRGINIA

MAY 10, 1944

**SOME ORGANOMERCURIALS AND THEIR BACTERIOSTATIC ACTION.** *F. J. von Gutfeld and W. A. Moomaw*, Medical College of Virginia.

It might be said that in general the most desirable characteristics of a bacteriostatic agent are effective action in a low concentration, low toxicity to the host, noncorrosiveness to tissue, noncapability of coagulating proteins, noncorrosiveness to surgical instruments and containers, and nonstaining ability (in specific cases a dye is desired). With these criteria in mind the literature was reviewed to learn the kind of substances which had already been investigated and to determine the character of those which should be considered further. It was concluded that the hydroxyphenylmercuric salts deserved further investigation, and attention was focused on the preparation of orthohydroxyphenylmercuric bromide, orthohydroxyphenylmercuric iodide, orthohydroxyphenylmercuric mandelate, and 1-hydroxy-2-nitro-4-(mercuric acetate) benzene for immediate study.

The organomercurials named above were tested against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The bacteriostatic activity was examined in 1 per cent peptone water and in 20 per cent human serum contained in 1 per cent peptone water, respectively. The presence of human serum usually diminished the bacteriostatic effect.

The ratio (bacteriostatic effect in 1 per cent peptone water: bacteriostatic effect in 20 per cent human serum contained in 1 per cent peptone water) yielded different figures with the bacteria tested.

**GLUCOSE UTILIZATION COMPARED WITH pH PRODUCED WHEN INFLUENCED BY CONCURRENT DECOMPOSITION OF PEPTONE BY THE GENUS BACILLUS.** *S. R. Bozeman and F. S. Orcutt*, Virginia Polytechnic Institute.

A lack of sparing action of carbohydrates on peptone by species of the genus *Bacillus* makes it difficult to determine the utilization of various carbohydrates as measured

by pH. The pH of the medium is dependent not only upon acid produced from carbohydrate dissimilation but also upon concurrent acid and ammonia from peptone decomposition.

The purpose was to determine glucose utilization by direct titration to separate it from the other two factors influencing pH change in the medium. It was found that by determining the pH potentiometrically and the glucose utilized (in the presence of peptone) by direct analysis that 131 strains and species of the genus *Bacillus* are separated into five groups.

In two of these groups the pH and glucose utilization are correlated, as would be expected—low pH with sugar utilization, and neutral or high pH with little or no glucose utilization. Another group, however, gave results quite unexpected—pH lowered to less than 5.5 even though little or no glucose was utilized and no acid produced from peptone. In a fourth group the pH was lowered only to the pH range of 5.5 to 6.5.

**FURTHER EVIDENCE DEMONSTRATING THE UTILIZATION OF GLUCOSE, SUCROSE, AND D- AND L-ARABINOSE BY CERTAIN MEMBERS OF THE GENUS *BACILLUS* IN WHICH THE pH OF THE SUGAR PEPTONE MEDIUM IS NOT APPRECIABLY LOWERED.** F. S. Orcutt, S. R. Bozeman, and W. B. Coffee, Virginia Polytechnic Institute.

It has been observed that a lowering of the pH in a sugar peptone medium is not a true measure of whether or not a species of the genus *Bacillus* is able to ferment the sugar present. Acid or ammonia from peptone, or both, interferes with a correct interpretation. It was concluded that correct data are obtained (1) when an indicator is used that will detect slight acid production; (2) when a peptone control is run concurrently with the fermentation; and (3) when fermentation is followed for a period of one week.

Even using these conditions it is difficult to be certain of sugar utilization in some cases. The present paper is concerned with the direct determination of glucose, sucrose, and d- and l-arabinose used in cases in which the pH does not fall below the neutral range for a period of one week, or is not significantly different from the peptone control.

It has been demonstrated that appreciable amounts of sugar may be used in such cases. This constitutes further evidence that much of the data in the literature regarding the ability of these bacilli to ferment sugars is in error.

**STUDIES ON THE TRANSMISSION OF "ATHLETE'S FOOT" BY SOAP: EXPERIMENTS WITH ARTIFICIALLY INFECTED FUNGUS CARRIERS.** F. J. von Guelfeld and R. E. Stone, Medical College of Virginia.

Experiments have been conducted regarding the possible transmission of a fungus infection (*Trichophyton*, *Microsporon*) to a healthy individual through the medium of a cake of soap infected by a person with "athlete's foot." Cotton strings and small squares of linen were infected with *Trichophyton gypsum*, *Microsporon audouini*, or *Microsporon lanosum*. These carriers were placed on cakes of soap (four well-known brands). The carriers were removed after varied periods of exposure, thoroughly rinsed with sterile distilled water, and transferred to 1 per cent peptone water. The experiments showed that all three fungi were viable after at least 60 minutes of contact with the soaps. These model experiments demonstrate that the possibility of the transmission of "athlete's foot" through the use of infected soap cannot be excluded. Tests with naturally infected human material are in progress.

**SHELLFISH INVESTIGATIONS IN THE LOWER CHESAPEAKE BAY.** Paul S. Galtsoff, U.S. Fish and Wildlife Service.

**STUDIES ON THE STABILITY OF BACTERIAL VACCINES.** F. J. von Guelfeld, Department of Bacteriology and Parasitology, Medical College of Virginia.

The bacterial suspensions used in World War I for the immunization against typhoid fever and cholera were insufficient in various technical respects: freshly prepared suspensions caused untoward by-effects, the transportation was rather complicated, and the usability of these vaccines expired after a relatively short period of time. I prepared in 1919 cultures of typhoid bacilli and cholera vibrios on agar. The bacteria were suspended in a small amount of

distilled water and killed by heat. The water then was evaporated by a current of sterile warm (37 C) air until a dry residue was obtained. The dried bacteria were pulverized, and the powder was kept in an ordinary sterilized glass bottle with a glass stopper. The dried bacteria were resuspended in sterile saline, their antibody-binding and antibody-producing properties compared with vaccines made in the usual way. The dried bacteria, tested a short time after drying, and again after 30 months' storage at room temperature, had about the same efficacy. Tests were performed 25 years after the bacteria had been dried. The suspensions of the dried bacteria were compared with vaccines which had recently been made. The dried typhoid bacilli exhibited agglutinin-binding and agglutinin-producing properties which compared with the corresponding properties of the fresh vaccines. The cholera powder had retained its agglutinin-binding activity; its agglutinin-producing property is still under trial.

THE RESPIRATION OF BACTERIA. *P. Arne Hansen*, U.S. Fish and Wildlife Service.

KILLING AND DISINTEGRATION OF MYCOBACTERIUM TUBERCULOSIS BY A VIRIDANS STREPTOCOCCUS IN VITRO. *F. S. Orcutt and A. W. Bengtson*, Virginia Polytechnic Institute and Catawba Sanatorium.

It has been demonstrated that when a viridans streptococcus is inoculated on an actively growing culture of *Mycobacterium tuberculosis* after a good growth of the acid-fast organism has taken place, the coccus spreads rapidly over the tuberculosis culture and progressively kills and disintegrates it

VIABILITY OF NODULE-FORMING BACTERIA ON INOCULATED LEGUMINOUS SEED AS AFFECTED BY STORAGE. *F. S. Orcutt and Alma L. Whitman*, Virginia Polytechnic Institute.

Inoculation of legume seed before sale to the farmer is not considered to be practical, probably because the meager literature on this subject indicates that the rhizobia do not remain viable on the seed for a suffi-

ciently long time to make commercial inoculation effective. In such experimental investigations the seed was sterilized by the use of mercuric chloride or chlorine which are very difficult to remove completely from the seed. The rhizobia subsequently inoculated onto the seed could hardly fail to be affected by the residual disinfectant, especially if the seed was stored.

Most legume seed appear to carry some natural inoculation, although not usually good nitrogen-fixing strains. Superior inoculation strains should be equally viable. The purpose of this work was to investigate the possibility of inoculating seed some time prior to use, the experimental procedure employed to be free from the use of disinfectants that have a tendency to remain for some time on the seed.

Results indicate that inoculated rhizobia may remain on the seed nine months in numbers exceeding those required for good inoculation.

AN OUTBREAK OF FOOD POISONING OWING TO STAPHYLOCOCCUS AUREUS. *George McL. Lawson and Thomas S. Englar*, University of Virginia School of Medicine, and Joint Health Department, Charlottesville.

Food served by a caterer to two groups was infected with *Staphylococcus aureus*. The first group was served within five or six hours after the preparation of the food. None were made ill. A portion of this same food eaten 54 hours after its preparation caused typical symptoms of this toxemia.

Food from the same source served 26 hours after its preparation caused illness in over two-thirds of those consuming it. The food concerned, chicken and homemade boiled salad dressing, was handled by a person who was a nasal and pharyngeal carrier of *Staphylococcus aureus*.

The tracing of this epidemic and the bacteriologic examination of the foods concerned were made possible by the prompt reporting of these cases to the Health Department.

REPORT OF A CASE OF LISTERELLA INFECTION IN A COW IN SOUTHWEST VIRGINIA. *Ray D. Hatch*, Virginia Polytechnic Institute.

A single case of encephalitis, occurring in a large dairy herd, is described. *Listerella monocytogenes* was isolated from the brain of the affected animal at necropsy. No other cases of *Listerella* infection are recorded in the state of Virginia. An attempt is being made to find the source of infection,

or reservoir of infection, in isolated herds. It is probable that this condition occurs frequently in this territory but is improperly diagnosed. The difficulty with which the organism is recovered from the infected brain contributes to the difficulty of diagnosis.

### CENTRAL NEW YORK BRANCH

SYRACUSE, NEW YORK, OCTOBER 28, 1944

**SUBSTITUTES FOR CARBON DIOXIDE AS AN ESSENTIAL FACTOR FOR THE GROWTH OF A STRAIN OF MENINGOCOCCUS.** *Henry W. Scherp and Dorothy M. Tuttle, University of Rochester, Rochester.*

The complete replacement of the supplemental CO<sub>2</sub> requirement of the meningococcus by 0.1 per cent yeast extract (Difco) suggested an investigation of individual constituents. A strain of meningococcus showing the greatest dependence upon CO<sub>2</sub> was tested in Frantz' synthetic medium.

Yeast nucleic acid was partially effective in an optimal concentration of 0.01 per cent. Adenine was generally ineffective, whereas guanine (0.004 per cent), uracil (0.03 per cent), or cytosine (0.03 per cent) were partial replacements for CO<sub>2</sub>; growth lagged markedly at 24 hours, and slightly at 48. The combination of guanine, uracil, and cytosine diminished the lag period and otherwise was completely equivalent to CO<sub>2</sub>. A total concentration of 0.003 per cent was only slightly effective, whereas 0.3 per cent was inhibitory.

Vitamin-free casein hydrolyzate (Smaco) (0.5 per cent) could also almost completely fulfill the CO<sub>2</sub> requirement. Lower concentrations were ineffective even when supplemented by guanine, uracil, and cytosine (0.003 per cent total concentration).

**EFFECTIVENESS OF SULFADIAZINE AND ANTIPERTUSSIS SERUM IN THE TREATMENT OF PERTUSSIS.** *William L. Bradford and Elizabeth Day, University of Rochester, Rochester.*

**ONE-THIRD FERMENTATION OF RAFFINOSE BY STREPTOCOCCI.** *C. F. Niven, Jr., and K. L. Smiley, College of Agriculture, Cornell University, Ithaca.*

A number of streptococci were found able to ferment only the terminal fructofuranose

portion of the raffinose molecule, leaving melibiose as a residual product. The majority of the cultures of *Streptococcus salivarius*, *Streptococcus equinus*, and members of the Lancefield group G ("minute") exhibited this property. The majority of the raffinose-fermenting cultures of *Streptococcus mitis* and all of the *Streptococcus bovis* strains tested were able to ferment raffinose completely.

With the use of an appropriate strain of *Streptococcus salivarius*, melibiose was isolated and chemically identified as a product of the fermentation of raffinose.

**CAPSULAR SWELLING OF ESCHERICHIA COLI BY ANTIPNEUMOCOCCUS SERUM.** *Elfriede Fendt, Meyer Memorial Hospital and University of Buffalo, Buffalo.*

A typical strain of *Escherichia coli* (indole-positive, methyl-red-positive, Voges-Proskauer-negative) was isolated from the lungs of a sixty-year-old woman who had died from pneumonia complicating bronchiectasis. Capsules could be clearly recognized on first isolation. After a few subcultures the capsules were lost. Passage through mice failed to restore capsule formation. The strain showed marked capsular swelling with pooled antipneumococcus types 10, 10A, 11, 13, 20, 29, and 31 rabbit serum. All other antipneumococcus sera failed to cause such a reaction. Attempts to determine the individual type serum responsible for this reaction were unsuccessful. A suspension of the organism was agglutinated up to dilution 1:40 by both horse and rabbit serum of the following pneumococcal types: 10, 10A, 11, 13, 20, 29, and 31. However, these sera caused no precipitation with the supernatant fluid of the broth culture of this strain. The capsular swell-

ing of this strain of *E. coli* by antipneumococcus rabbit serum parallels the cross reaction between a strain of *E. coli* and pneumococcus type I reported by Barnes and Wight in 1935.

RELATIONSHIPS WITHIN THE GENUS RHIZOBIUM, AS SHOWN BY LYSIS WITH BACTERIOPHAGE. *H. J. Conn, Elizabeth J. Bottcher, and Challiss Randall*, New York State Agricultural Experiment Station, Geneva.

In several fields of bacteriology attempts have been made to use the specificity of bacteriophage to separate species of bacteria from one another, but without great success. Laird (Proc. World's Grain Exhib. Conf., Canada, 1933, 362) tried to apply the technique to the legume nodule organisms but found such variation in susceptibility as to make the method seem unpromising. The present writers decided to see whether more promising results could be obtained in this genus by using the bacteriophage technique of Hofer and Campbell (J. Bact., 45, 406). Such proved to be the case; results were reasonably constant and clear-cut. Almost perfect cross lysis was observed between strains and phage filtrates among the pea, bean, and clover organisms, often regarded as constituting separate *Rhizobium* species. The organisms of alfalfa, lima bean, soy bean, and other less well-known legumes did not cross-lyse with one another or with those of pea, bean, or clover. These results show close relationships among the pea, bean, and clover organisms, and indicate the possibility that the three constitute a single species.

THE OCCURRENCE OF CLOSTRIDIUM BOTULINUM IN SOILS OF CENTRAL NEW YORK. *E. Winifred Parry*, Syracuse University, Syracuse.

STUDIES ON THE COMPLEMENT FIXATION TEST AS APPLIED TO BOVINE TUBERCULOSIS. *Richard B. Johnson*, Veterinary College, Cornell University, Ithaca.

SUBSTRATE SPECIFICITY FOR THE TYROSINE DECARBOXYLASE SYSTEM OF STREPTOCOCCUS FAECALIS. *W. D. Bellamy*, College of Agriculture, Cornell University, Ithaca.

A FUMARATE REDUCTASE SYSTEM IN LACTIC ACID BACTERIA. *I. C. Gunsalus*, College of Agriculture, Cornell University, Ithaca.

Certain lactic acid bacteria have been found to ferment glycerol only aerobically, whereas other strains are able to produce acid from this substrate under anaerobic conditions (Gunsalus and Sherman: J. Bact., 45, 155). When a characteristic strain of the latter group was studied in detail, yeast extract was found to contain hydrogen acceptors which permitted the formation of lactic acid as the main product of glycerol fermentation. On further fractionation of the medium, a higher level of riboflavin was found to be required for glycerol fermentation than for glucose fermentation. In the presence of 1 per cent tryptone and added flavin, the yeast extract could be replaced by fumaric acid as a hydrogen acceptor. When the medium contained glycerol plus fumaric acid, the final products of fermentation were found to be lactic and succinic acids.

Preliminary studies have failed to show succinate oxidation by this culture. This appears to be another case in which a substrate which is reduced during metabolism is not readily oxidized by intact cells.

THE DESTRUCTION OF VITAMINS AND PIGMENTS BY NITROUS ACID. *J. K. Wilson*, College of Agriculture, Cornell University, Ithaca.

# CYTOPHAGA COLUMNARIS (DAVIS) IN PURE CULTURE: A MYXOBACTERIUM PATHOGENIC<sup>1</sup> TO FISH<sup>1</sup>

LAURA GARNJOBST

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A rod-shaped bacterium capable of unusual motility was described by Davis in 1922 as the cause of a distinct dermal disease of numerous fresh-water fishes. He named the organism *Bacillus columnaris*, but owing to changes in the system of classification since that time (Bergey *et al.*, 1939) it was automatically excluded from this genus. There is at present also a better understanding of other forms having this peculiar creeping mode of locomotion without flagella. The vegetative rod form, thin cell membrane, flexibility, and the aflagellar progressive movement on surfaces now indicate the complex (higher) myxobacteria or closely related species.

It is the purpose of the present paper to give a more extended account of this interesting and important parasite—an account made possible by its isolation and cultivation in a variety of media. Special emphasis has been given to the study of its life cycle, but no fruiting bodies or microcysts appear to be formed. Therefore, the bacterium has been assigned to the family *Cytophagaceae* (Stanier, 1940), genus *Cytophaga*. If this position is accepted, *columnaris* becomes the first member of the *Cytophaga* group (Stanier, 1942a) known to be an animal parasite. Its nutritional requirements consequently should be of general interest.

## TECHNIQUE

The initial colonies of *C. columnaris* were obtained from infected bullheads (*Ameiurus nebulosus*) taken from a pond at the Leetown laboratory of the U. S. Fish and Wildlife Service in July and August, 1943. To eliminate most of the other, usually more rapidly growing, bacteria from the original inoculum, the following method was found best. A flake of debris was removed from a selected area of the lesion, deposited at one side of a large drop of sterile spring water on a slide, and left until the rods had swarmed away from the mass for some distance. The debris was then removed with a pipette, and some of the remaining bacteria were transferred with a loop in the usual way without further dilution.

Colonies appeared both in nutrient gelatin and in nutrient agar plates in four or more days at temperatures between 13 and 25 C°. In all, 15 colonies were removed from semisolid nutrient gelatin into the following liquid media: (1) fish extract with 1 per cent peptone added, (2) Difco proteose peptone (1.0%, 0.25%); (3) tryptone (0.5%); and (4) proteose peptone containing hemoglobin (0.5%). Growth occurred in all except medium (1) but very little, if any, in (1). Four of the original cultures from these colonies probably were pure from the

<sup>1</sup> Published by permission of the Director, U. S. Fish and Wildlife Service

beginning, since serial transfers were made at irregular intervals for six months without contaminants making their appearance. Strains from five colonies were further purified and maintained so, but no significant difference has been noted between them, or between them and the strains not further purified, in behavior or appearance of the bacteria during the period of study (August, 1943, to February, 1944).

The pathogenicity of the bacteria grown in pure culture was tested by inoculation into slight surface injuries in healthy sunfish. Within certain temperature ranges, the characteristic lesions of this and other kinds of fishes in natural and induced "columnaris infections" were readily produced, followed by death of the fish in 100 per cent of the cases (Davis, report in preparation). The bacteria in culture also retained their distinctive behavior, including the columnar swarming movements in water mounts. For these reasons there is no doubt that the organism isolated is the same as the long flexible rod found in abundance and in almost pure culture in the lesions of fish.

Stock cultures were maintained in a 0.5 per cent proteose peptone or tryptone solution (initial pH, 6.3 to 7.1). The bacteria were sometimes difficult to remove from an agar surface after about 24 hours of growth and, therefore, a liquid medium was preferred, especially in the early stages of the study. Each strain was maintained at two different ranges of temperature, 13 to 18 C° and 20 to 25 C°. Some of the tubes at the lower temperatures remained viable for more than 30 days.

Preliminary experiments were made in an effort to obtain an entirely adequate stock medium. Eight different media, including solutions of peptones, yeast, and *Elodea* extracts were prepared at optimum concentrations previously ascertained and the amount of growth obtained compared. Of these, proteose peptone and tryptone were selected as suitable basal media (pH about 6.8) for testing other substances which might be added. Small amounts of glucose or lactose added did not appear to give more rapid or extensive growth. Yeast extract added to tryptone solutions (known to be low in vitamins) delayed the appearance of turbidity as compared with the controls. A solution of vitamins (thiamin, nicotinic acid, calcium pantothenate, riboflavin, *para*-aminobenzoic acid, inositol, and pyridoxine) with or without glucose did not produce any noticeable effect, but there was no period of lag at the beginning.

The bacteria were fixed and stained in a variety of ways from several kinds of media. In addition to the usual routine bacteriological methods, cytological procedures without drying at any stage were adapted to the organism. Zenker's or Worcester's fixing fluids gave good results, followed by staining in hematoxylin (Heidenhain's or Ehrlich's) or basic fuchsin. Other fixatives tried out were Bouin's, Schaudinn's, and Da Fano's cobalt nitrate. Differential staining methods used, in addition to Gram's, Neisser's acid methylene blue and Bismarck brown, and Epstein's granule stains, were Mallory's tricolor after Zenker's fixing fluid, Giemsa's dry, and Dobell's (1911) wet method using Bouin's fixative, Winogradsky's (1929) phenol-erythrosine and gentian violet after fixation in osmic acid fumes, and finally Robinow's (1942) modification of Feulgen's "nu-

clear" reaction. These methods proved useful from a comparative viewpoint, especially taking into account the structure as observed in the living bacteria.

Other techniques and staining methods are mentioned in connection with the structure or experiment concerned. Unless otherwise stated, all measurements given were made on living material.

#### MORPHOLOGY

The most common form of *C. columnaris* observed in nature is the long, slender, flexible rod, with rounded ends, 5 to 12 microns long and about 0.2 microns wide (fixed and stained cells), first described by Davis (1922). Three other forms were discovered in pure culture: (1) long filaments, segmented or unsegmented, (2) branched cells, and (3) "ring forms," simple and complex. This study has also extended the dimensions. Filaments were found which measured as high as 163 microns in length, and the smallest cells were only two microns long. The vegetative cells (figure 1, nos. 1 to 8; figure 2, nos. 46, 48) in peptone media averaged about 8.0 microns, width measurements varied from 0.2 to 0.4 microns. On nutrient agar these cells averaged about 10 microns in length. None of the rods tended to appear spindle-shaped at any time.

Multiplication is by transverse division, usually into two cells of approximately equal length. The division appears to be by cell "constriction" (Stanier, 1940, 1942a); in wider cells, such as *Cytophaga rubra*, this detail undoubtedly is more readily determined. In living material, recently separated cells sometimes seem attached to one another by a connecting thread, but it is difficult to be certain that the strand is not secreted slime. Before separation of the cells, the rod often becomes bent at the mid-point. Such V-shaped structures were everywhere in evidence in young fluid cultures, but they also appeared when cells near the period of elongation were brought into water from an agar plate culture. The change seemed to stimulate cell division, and the whole process till complete cell separation could then be observed under the microscope repeatedly. It is not meant that cells necessarily always bend in this manner prior to separation, but it is very characteristic of this species. It is not accidentally brought about, for instance, in transfer, but rather by movement of the cells themselves (see section on movement).

The filaments are unsegmented or segmented, depending upon the time when they were removed from a culture. In older cultures cell division as well as cell separation appears to be suppressed. Upon transfer to a fresh medium the unsegmented filaments often become segmented and separate into cells, usually of unequal length at least at first, the final result being cells of small size (2.0 to 5.0 microns). Under circumstances of more or less precipitous division of filaments, branched cells were first noted (figure 1, nos. 9 to 14; figure 2, no. 49). The dense layer of filaments lying parallel to one another, as they generally are, would of course be more durable with branched than with unbranched filaments alone. If they are involution forms they nevertheless have been seen undergoing flexion and in creeping movement. Two or more branched forms in one field of the microscope were not uncommon in some unstained as well as stained preparations.



Krzemieniewska (1930) described similar branched cells in *Spirochaeta cytophaga*<sup>2</sup> and found them to have nothing in common with the branched cells of the actinomycetes.

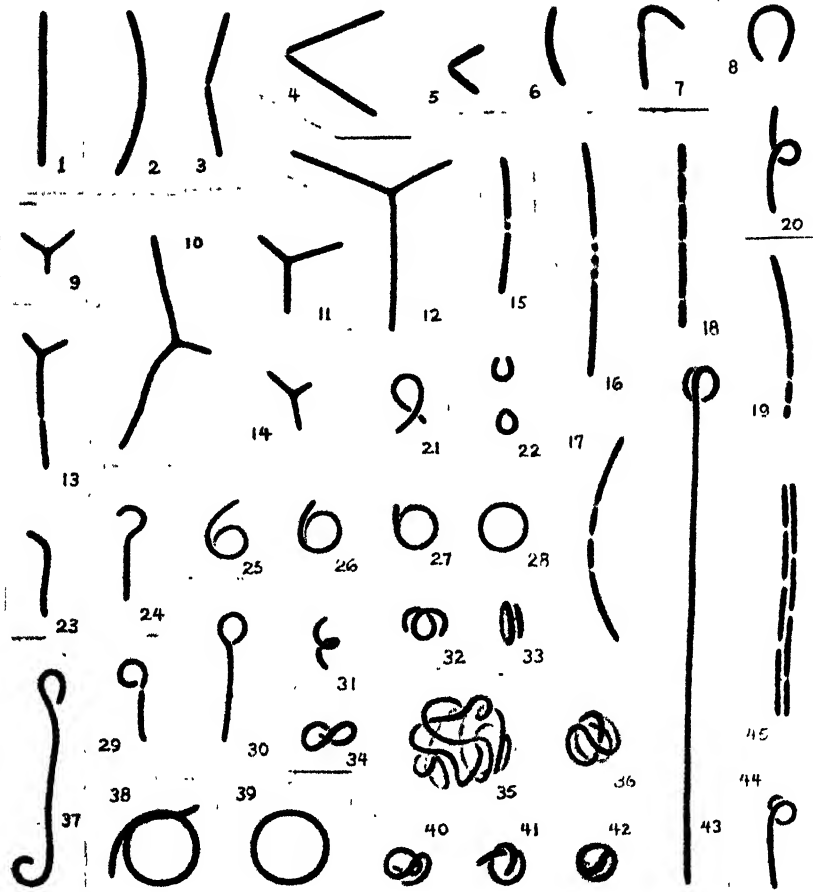


FIG. 1

All drawings were made with the aid of a camera lucida

Nos 1-45 incl *Cytophaga columnaris*. Drawings made from fixed and stained preparations except as noted below. Bacteria grown on peptone nutrient agar.  $\times 2600$ , reduced  $\frac{1}{2}$ .

Nos 1-8 Vegetative cells, from living material. Nos 3-5, stages of division.

Nos 9-14 Branched cells, from living material. Nos 11-12, from living material.

Nos 15-19. Pseudobarred or beaded cells (probably artifacts).

Nos 20-44. Coil or ring involution forms. Nos. 20-27, from living material. No. 35. Complex ring form, composed of one long filament. Nos. 35-43, cells stained with vital neutral red

No. 45. Section of two segmented filaments

*C. columnaris* cells do not retain stains as readily as many other bacteria, although after fixation in Zenker's fluid and staining for several hours in basic

<sup>2</sup> *Sporocytophaga myxococcoides* (Hutchinson and Clayton emend. Krzemieniewska), Stanier (1940).

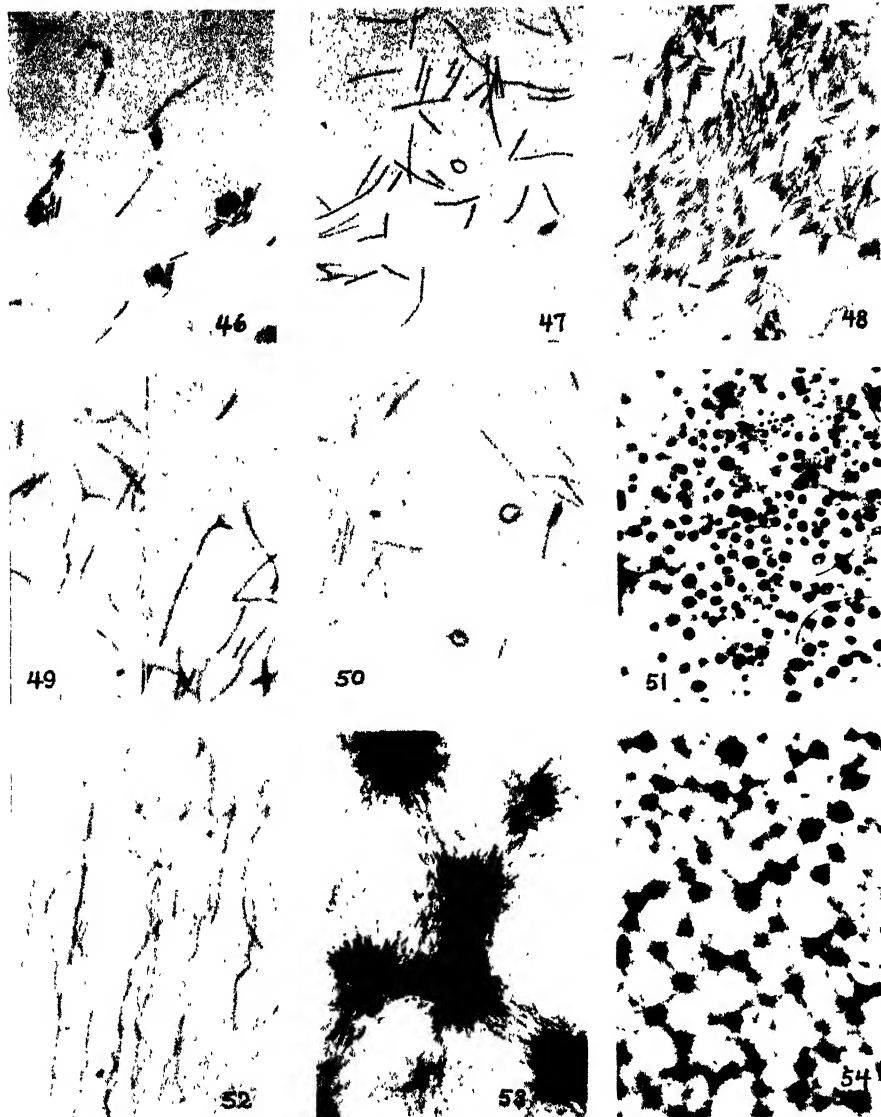


FIG. 2 PHOTOMICROGRAPHS OF FIXED AND STAINED  
*Cytophaga columnaris* PREPARATIONS

Nos. 46 and 48. Vegetative cells from surface of agar plate culture. Zenker's fluid, basic fuchsin.  $\times 680$ .

No. 47. Vegetative cells from peptone agar showing one simple ring form. Worcester's fluid; basic fuchsin.  $\times 680$ .

No. 49. Two branched cells from 0.5 per cent tryptone. The larger cell is in an early stage of division. Worcester's fluid; basic fuchsin.  $\times 1075$ .

No. 50. Coiled rods. Zenker's fluid; Ehrlich's hematoxylin.  $\times 1075$ .

No. 51. Late stages of coiled cells (ring or coccus involution forms), with a few rods present. Worcester's fluid; Heidenhain's iron-alum hematoxylin stain.  $\times 680$ .

No. 52. Long, undulated involution forms from an old culture. Worcester's fluid, Heidenhain's iron-alum hematoxylin stain.  $\times 1075$ .

Nos. 53 and 54. Pellicle removed from an agar surface culture, showing pattern formed by the bacteria. Note the arrangement of bacteria about the clusters. In no 51 in the center of the larger clusters can be seen a thicker patch of slime to which the rods adhere, the photograph was taken at a level near the base of the clusters. No 51  $\times 340$ ; no 52,  $\times 170$ .

fuchsin, more deeply stained rods were obtained. The differential staining methods, such as Robinow's modification of Feulgen's technique, Winogradsky's stain, and Heidenhain's iron-alum hematoxylin, revealed no internal structures.

Invariably the living as well as the stained cells are homogeneous in appearance. This was found to be true also by Davis (1922). No colored particles appeared after application of scarlet R or Sudan III in 80 per cent alcohol. Also, no color reaction could be obtained in the living cells with the vital stains methylene blue or neutral red. On the other hand, the dilute solutions of these dyes stained the secreted substance vividly, producing a beautiful effect—colorless, living, moving cells adhering, often only at one end, to a bluish-purple or red substance, depending upon which stain was used. A test for volutin described by Meyer (1912), in which cells are first stained in methylene blue and then treated with 1.0 per cent sulphuric acid, left no blue color within the cells.

Some cells fixed by drying have a barred or segmented appearance, but this is not comparable to the transverse bands described by Krzemieniewska (1930) since in *C. columnaris* there is no difference in stainability. The protoplasm within the cell membrane is changed so that it appears to be segmented or beaded as shown in nos. 15 to 19 (figure 1). It is often noted that one to three beads (that is, granules extending the width of the cell) are present at the mid-point of a rod likely to be near division. Cells from the same source stained (upon death) with vital methylene blue or neutral red or fixed in the sublimate mixture, Zenker's or Da Fano's fluids, show less distinct but similar structural details in some cells. However, preparations made by pressing a coverslip on the cells growing on an agar surface and fixing the readily adhering cells in Zenker's fluid promptly without drying preserved most nearly the conditions of the living cell (figure 2, nos. 46, 48), and in these the structural effects just described are usually absent, although normal (that is, at least viable), segmented filaments occur. It is suggested that the pseudobarred or beaded conditions probably are produced at the time of fixation, and more often if the cells are near division (single or multiple). The protoplasm at this period (immediately before and after division) might be more fluid generally throughout the cell or at certain points. None of the differential stains tried produced differences in color or in intensity of stain between any such granules or segments present. The possible formation of conidia was considered.

Under certain not specifically known circumstances rod-shaped cells become irreversibly coiled (figure 1, nos. 20 to 44; figure 2, no. 50). Although it has long been known that a myxobacterial cell can bend, for instance in the form of a ring, Stapp and Bortels (1934) were the first to suggest that the peculiar "Krümmungsbewegungen" might be pathological in character. Stanier (1942b), on the other hand, stated emphatically that the flexing movements are not symptoms of degeneration, as believed by Stapp and Bortels, but are "always of very marked occurrence in the majority of young, healthy cells which are lying in contact with a surface" (p. 155). In *C. columnaris* normal flexing movements occur and were, indeed, very often observed (see section on movement), but these do not preclude irreversible coiling of cells. The usual fate of cells which remained coiled

appeared to be a series of involution or degenerate stages, from distinct, readily recognizable ring forms (simple or complex) to rounded bodies of various sizes, depending on the size of the cells which coiled, and finally to amorphous granules. After coiling, a veil of secreted slime often became associated with the cell so that, especially in the simple rings and in later stages of more complex rings, the whole presented the appearance of a coccus-shaped cell (figure 2, no. 51). One cannot escape the impression that it would be easy to confuse these stages with microcysts. They stain deeply in hematoxylin, and after the readily recognizable coiled stage the deeply staining part is irregularly distributed. Finally, in the irregular granules, stain is no longer retained to any great extent. The staining property of these involution forms call to mind the so-called pycnotic nuclei and their later stages of degeneration.

Coiling could be readily induced by mounting bacteria from liquid media or an agar surface in water. With or without a coverslip and usually within about ten minutes, coiling took place. In one such instance under a coverslip as many as 20 to 40 rings could be counted in any microscopic field. This number, however, was uncommon in preparations without a coverslip added. The degenerative stages sometimes were reduced so that within about one hour nothing remained but small homogeneous-appearing droplets, and these stained deeply in hematoxylin.

Coiling is not a function of a definite stage; but it appears rather to be due to certain environmental conditions. Cells of various sizes and cells undergoing division were observed to coil; even very long cells produced ring forms, and these were large and often complex (figure 1, nos. 35, 36).

Whatever their meaning the ring involution forms are important in a study of this kind: they are conspicuous and, as stated, the coccuslike stages could easily be confused with microcysts. Their mode of formation, however, is entirely different from that described for microcysts (Baur, 1905; Krzemieniewska, 1930; Stanier, 1942a; and others), for there is no shortening of rods to form the coccus stage. The presence of a few simple rings is very common, but in only one tube, containing 0.5 per cent of tryptone, did practically all the cells assume various simple and complex ring forms. When the coccuslike forms were found in great abundance, there were invariably present also at least a few rods. One exception observed was a plain gelatin culture. Experiments were made to test viability and these are described below.

#### MOVEMENT

Studies have been made of the movements of myxobacteria, notably by Baur (1905) and Stanier (1942b); certain differences are stressed in the following brief account of movements in *C. columnaris*. The bacteria were observed in water mounts and on nutrient agar surfaces. In a liquid nutrient medium on a glass slide, movements were rarely seen unless the nutrients were very dilute.

The rate of creeping locomotion in water on a glass surface (with the temperature uncontrolled but approximately between 20 and 22 C°) was found to be relatively high (12 to 94  $\mu$  per min; average of 14 readings, 60  $\mu$  per min) as com-

pared with that on nutrient agar, and as reported for the marine species ( $30\ \mu$  to  $50\ \mu$  per min). In rate of movement, *C. columnaris* resembles the soil cytophagas in which the rate has been estimated to be approximately  $150\ \mu$  per minute, without temperature control (Stanier, 1942a). The rate, however, varied greatly from barely perceptible to rapid.

In following the course of a cell one might see it glide over other cells, stop at times, and then resume movement, usually in the same direction, although the course was not necessarily in a straight line. The direction of movement was occasionally reversed (1) by creeping with the opposite end foremost, for instance upon contact with an obstruction, or (2) by a swinging movement (that is, the rod taking suddenly a perpendicular and then a horizontal position), the same end being foremost in the resumed gliding but pointing in the opposite direction. On a moist agar surface such freedom of movement was probably prevented, but flexion was possible so that the direction was occasionally reversed (3) by bending.



FIG. 3. COLUMNAR MOVEMENTS ON NUTRIENT AGAR SURFACE  
Zenker's fixative; basic fuchsin.  $\times 2600$  reduced  $\frac{1}{3}$ .

Permanent records (figure 3) of movements of this kind were obtained when a coverslip was pressed on the bacteria swarming on an agar surface and the whole straightway immersed in Zenker's fixing fluid.

Movements in water were of special interest. In addition to those already described, peculiar rotary or waving movements, sometimes combined with flexion, were observed in rods which had suddenly assumed a perpendicular position on glass. The waving was so regular that automatically one began to count. The result of counts made on several different individuals are: 111, 218, 193. The horizontal position was suddenly resumed and sometimes just as suddenly the rod became perpendicular again to repeat the process. Usually the rod remained fairly rigid during the movement. On the other hand, one cell was less rigid, and in this the beat was irregular and rapid so that accuracy in counting the revolutions was impossible. The circle described by the free end was not complete and the rate dwindled noticeably just before the cell dropped to the horizontal position. These movements, which seem fantastic in so simple an

organism, are nevertheless fairly similar to those often seen when the bacteria emerge from a mass taken from a fish lesion or from an agar surface, but in these isolated individuals it was much easier to study them. Of all the movements observed this type is the only one which would seem to require flagella, but flagella were not discovered by special staining methods, such as Loeffler's and Fisher and Conn's, or relief stains using nigrosine or China blue. Since the electron microscope has revealed flagella in a spirochete, *Spirocheta pallida* (see Marton, 1943), the question should perhaps be left open.

It was interesting to observe cells in active movement undergoing division. When the rod became bent to a V-form, the movement became confused, and this undoubtedly aided in further bending and separation of the two cells. In one instance it was always one cell which moved; the other was bent above the moving cell and so carried in the same direction. The movement was even more confused when a longer rod became bent in two places. It is not impossible, though to the present investigator rather unlikely, that V-formation can occur in the absence of division. Benecke (1912) stated as follows: "... die normalerweise gerade, stäbchenförmige Myxobakterienzelle kann sich kreisförmig biegen oder auch zusammenknicken . . ." (Stanier, 1940, p. 620), an indication that the phenomenon has been observed in other myxobacteria. Baur (1905) in fact described it but did not associate it with fission.

The longer the cells become the less likely are they to be seen in motion. Even so, cells 20 microns long without any signs of division were observed to creep rapidly and characteristically on glass in a water mount. Long cells in a filamentous mass were seen undergoing a restless, rotary type of motion in entirely undisturbed areas of an agar plate.

#### CULTURAL CHARACTERISTICS

*Liquid media.* In the vegetative stages the rods appear rigid (turgid?). When not in movement they are straight or slightly curved (figure 1, nos. 1, 2) and readily cling to any small particle present, or to one another. The resulting clusters or stars, and individual cells as well, are distributed throughout the medium. In some instances clusters were found attached to the side of the tube where they formed distinctive colonies (figure 4), superficially resembling minute sea urchins. They ranged in size from microscopic dots to visible objects needing no magnification. In older stages the cells swarmed from the enlarged, rounded, or irregular mass, with the result that often papillae-like clusters projected from the entire exposed surface. These projections varied in number and eventually elongated into columns of organisms; or, rounded tips were formed which became detached into the medium as spheres.

A surface film was formed in some tubes, and there was considerable piling up of cells at the bottom, particularly at the lower temperatures (13 to 18 C°). In such tubes, when shaken, a large spiral formed from the bottom layers, as would be expected, since the strands are composed of cells (some of them long) adhering to the glutinous secretion. A distinct yellow color often developed on the surface film or in flakes of declining cultures. A yellow ring was formed in fish extract.

Some of the bacteria, both long and short, were coiled into rings, especially at the higher temperatures, and masses of these were found in various stages of degeneration. Other remaining cells seemed finally to have lost their turgidity (involution forms), for they were twisted in any manner and undulated (figure 2, no. 52), and the threads were noticeably thicker.

*Solid media.* *C. columnaris* grows fairly well on an agar surface, such as Difco nutrient agar. The surface pattern formed by the bacteria varied, of course, depending in part upon the consistency of the agar and the amount of moisture present in the air above, or below, the surface. If the agar was firm and little moisture present in the air, no growth occurred at all. Just the right

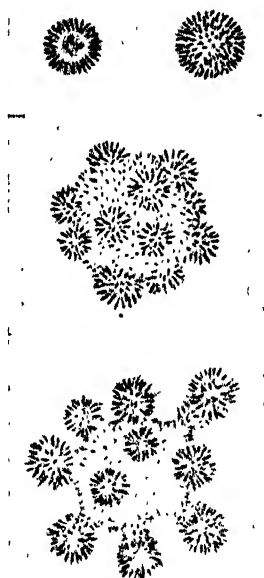


FIG. 4. CLUSTERS (OR STARS) FORMED IN 0.5 PER CENT TRYPTONE SOLUTION  
Living material. Semidiagrammatic.  $\times 125$ .

amount was required. Until this was realized, the growth response of the organism on agar (and gelatin, to some extent) seemed erratic.

Within the first 24 hours of growth following surface streaking from a liquid culture, a pellicle was readily removed (peeled) from the agar. This pellicle<sup>3</sup> was uneven in thickness and the pattern formed by the bacteria adhering to it was related to the movement of the organism on a solid nutritive substrate. After 24 hours, more or less, the pellicle usually became closely adherent to the agar and could not be successfully removed. The bacteria as they migrate from a

<sup>3</sup> The pellicle, composed of bacteria and the yellowish secretion, gives a blue color reaction with concentrated sulphuric acid. Small flakes almost immediately are haloed by a bright blue color which increases in extent. In a few minutes the blue color begins to fade into a dirty yellow and then becomes distinctly reddish in color.

given point both tend to spread over the surface in columns and to form aggregates or clusters (figure 2, nos. 53, 54) from which other migrations occur if there is considerable moisture present; otherwise, the aggregates remain small and branching, interlacing columns are formed. Variations between these two extremes of pattern occurred also. The layers of bacteria increase because the organisms continue for some time to migrate back and forth and to multiply along the "paths" taken. If the inoculation of the agar surface has been such that colonies were well separated, the original points of growth were recognizable for many hours after the whole surface of the plate had been overgrown with bacteria.

Samples removed from a plate about 18 hours after inoculation usually showed elongated cells in some portion of the growth. These gradually increased in number until there was formed a more or less continuous, felted cover with many of the filaments lying in parallel rows or swirls. When samples were removed during the early period of elongation and mounted in water, the cells invariably began swarming from the mass in precisely the same manner as that described and figured by Davis in samples taken from lesions of fish. Samples were then taken at hourly intervals for 10 hours, and it was discovered that finally this behavior no longer took place. The cells had become long. When transferred to nutrient media they reverted to small cells, but not in all cases. Just when the filaments become nonviable (that is, when no further reproduction occurs) is not readily determined, since it is difficult to test many individual filaments. If all rods which divide into more than two cells are involution forms, they may nevertheless be of some importance to the species. It is possible that the suddenly increased numbers at high temperatures in ponds or streams may be enhanced by the multiple division if at the same time nutrients are available.

In declining agar plate culture, the color *en masse* becomes changed from pale yellow to a deeper, old-gold shade, sometimes with a tinge of orange. Samples taken showed rings and ring involution forms in patches. A characteristic sickening odor is given off by growth of the bacteria on Difco nutrient agar, and this was most noticeable after 24 hours or more. No reference to an odor of this kind was noted in the literature regarding the myxobacteria.

To test the presence of resistant stages, inoculations into liquid media were made daily from an agar plate culture. After 48 hours, the inoculum contained elongated cells and rounded bodies. With time, the former decreased in number whereas the latter increased in number. The time to the appearance of turbidity in tubes of tryptone solution increased, and after the eighth day the solution in the inoculated tubes remained clear. This experiment was repeated with rounded bodies formed in nutrient gelatin with the same result in seven days. In one tube rounded bodies only were present in the gelatin. No growth was obtained in the tubes inoculated. Both unstained (living) and stained preparations made from this and similar material showed that the structure of the rounded bodies resembled in every way later stages of ring involution forms.

*Colonies.* Young surface agar colonies (figure 5) are flat, colorless or pale yellow, and intricately branching. In the spreading outer zone, particularly, the



branches often appear discontinuous owing to the swarming of cells in groups. The pattern in the peripheral zone for some distance is continually modified. Branches are sometimes withdrawn, and an entirely new pattern appears; or a group of rods becomes stranded at a distance, sometimes far enough to begin a new colony. The older, central portion of an old colony formed from a minute

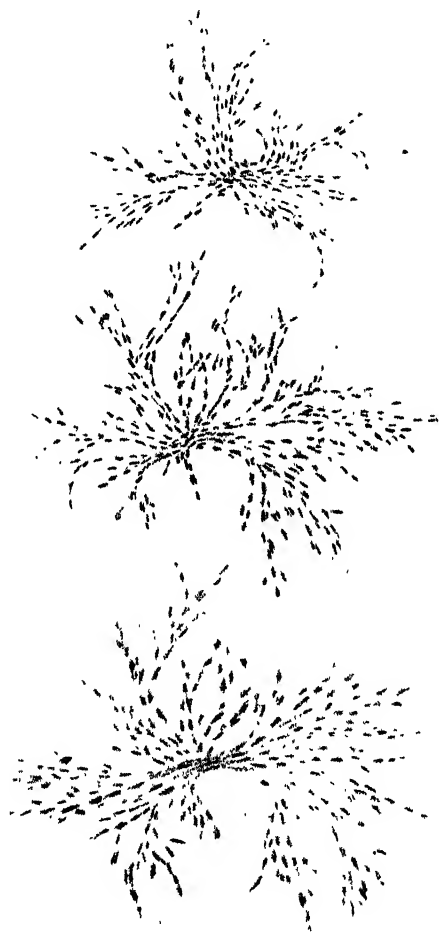


FIG. 5. SURFACE PEPTONE AGAR COLONIES (72 hr.)  
Semidiagrammatic.  $\times 250$ , reduced  $\frac{1}{4}$ .

inoculation at the center of an agar plate becomes warty in appearance, and the pattern of the surrounding zone gradually becomes fixed into layers of long filaments along the main branches. These colonies rarely exceed 5.0 cm in diameter. Colonies beneath the agar surface are massive, and globular or irregular. Sometimes projections are present, their formation depending upon the softness of the agar and the consequent migration of the cells if enough moisture is present. All

gradations of agar colonies were observed from entirely globular colonies to a condition in which a central globular mass was surrounded by the characteristic flat network of branches. In older stages the network was sometimes withdrawn, leaving a deep yellow, entirely spherical, surface colony.

Gelatin colonies resembled those formed on agar, but the bacteria soon liquefied the gelatin and therefore the colonies became irregular, filamentous masses. These colonies were readily removed *in toto* because the cells adhered to the slime secreted.

#### NUTRITIONAL REQUIREMENTS

*Nitrogen.* *C. columnaris* was found to grow satisfactorily in a medium containing only peptone. This is not surprising when one considers its luxuriant growth on the dermal cells of fish at optimum temperatures, 25 to 30 C°. Experiments (at 25 C°  $\pm$  0.5) to test whether or not the nitrogen requirements could also be met by supplying an inorganic source were negative. KNO<sub>3</sub> or (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> were supplied in concentrations ranging from 0.1 to 1.0 per cent in combination with 0.1 per cent glucose or lactose in Leetown spring water or the mineral medium used by Stanier (1942). The only growth which occurred was in the control tubes which contained also 0.1 per cent peptone, and this was of the order found in a 0.1 per cent solution of peptone alone.

Peptones are not required as such, however, since the organism will grow well, even after several transfers, in a mineral base to which hydrolyzed casein has been added. These experiments also showed that the organism probably synthesizes any necessary vitamins, because the hydrolyzed casein used was found to be free of vitamins. (For this preparation the author is indebted to Dr. E. L. Tatum, Stanford University, California.) Further experiments are now being made to discover which amino acids are required for growth.

*Carbohydrates.* Preliminary experiments using the ordinary standard methods had shown no clear-cut positive results for the utilization of sugar, although in tryptone solutions the organisms could tolerate a concentration up to 0.7 per cent of autoclaved glucose without atypical growth. As previously described, a dilute solution of neutral red produced a definite color change to red in the slime secreted by the bacteria. Any consideration of acid production in small amounts would have to take into account this apparently basic substance produced. Using glass filter-sterilized glucose in amounts ranging from 0.01 to 0.1 per cent in 0.1 per cent peptone, no detectable utilization could be demonstrated by qualitative methods.

Agar appeared not to be decomposed in any recognizable degree. The I-KI solution test recommended by Stanier (1941) also was negative. The addition of calcium carbonate to an agar nutrient medium did not give more extensive growth than a similar agar medium without this substance added. The cellulose decomposition tests were made with filter paper strips in (1) the mineral medium of Stanier, with an initial pH of 7.1, and (2) in media containing 0.1 to 0.5 per cent peptone, or 0.1 per cent peptone and the nitrate salts (0.1%). In (2) there was no evidence of decomposition microscopically within 17 days. Although no

growth of *C. columnaris* was obtained in (1) within 14 days, *Sporocytophaga myxococcoides* (from Dr. C. B. van Niel) inoculated into the same medium produced evidence of growth in six days and macroscopic decomposition of the cellulose on the tenth day after inoculation.

#### LIFE CYCLE AND SYSTEMATIC POSITION

In the classification of the *Myxobacteriaceae*, as revised by Stanier (1942), great emphasis has been placed on the production or absence of microcysts in the life cycle. There is a tendency in the literature, moreover, to regard descriptions without this stage as incomplete, which is not surprising in view of work in other fields and the long known life cycle of the higher myxobacteria. The spores in yeasts, for example, are not readily obtained in culture, and a medium used to produce spores in one species does not necessarily produce them in others (Henrici, 1930), yet this stage has been selected as a means of classification. Studies on the higher myxobacteria have shown that fruiting bodies and microcysts are often discovered under natural conditions and sometimes fail to form, or are less normal, in the same species in certain laboratory media (Beebe, 1911; Snieszko, McAllister, and Hitchner, 1943; and others). Although no such stages have been found in *C. columnaris* under natural conditions or in the media already described, in the present study it was considered important to make further tests using complex media.

Fish tissue autoclaved in small amounts for a minimum of time (5 to 10 minutes at 121 °C), with or without agar, failed to produce microcysts in *C. columnaris* at several temperatures, including 30 °C. Several kinds of eubacteria isolated from pond water (*Pseudomonas* and *Sarcina* species) were autoclaved and added to the medium or used alone in agar or water suspensions, according to the methods described by Beebe (1941), but these also failed to give positive results.

Snieszko *et al.* (1943) suggested that the pronounced proteolytic properties of myxobacteria were probably responsible for the observations made by other investigators and themselves that when the vegetative growth is more profuse, there is a greater probability that fruiting bodies will not develop at all, or will be autolyzed before development is complete. *C. columnaris* forms proteolytic enzymes, known from its rapid liquefaction of gelatin, clearing action in milk, and slow dissolution of autoclaved bacteria. In regard to the latter it may be mentioned that the organism will grow in a medium composed of insoluble material prepared from *Photobacterium* sp. (for preparation see Tatum *et al.*, 1942) in a mineral solution. Scantier growth which permitted development in spite of enzyme production in two species of *Myxococcus*, according to Snieszko *et al.*, did not bring forth fruiting bodies or microcysts in *C. columnaris*. In tryptone or proteose peptone media with 0.1 per cent agar *C. columnaris* produced a heavy pellicle, but no fruiting bodies were formed. Ring involution forms were abundant.

There are now at least six (probably nine) myxobacteria in which microcysts are definitely considered absent (Stanier, 1942; Fuller and Norman, 1942). The present species differs from these cytophagas in several respects, the most important being that cellulose is not decomposed to any recognizable degree. This

statement leaves out of account two soil cytophagas, very briefly described by Fuller and Norman (1943), which also do not attack cellulose; but the fact that *C. columnaris* is an animal parasite probably would distinguish it from these soil forms. These differences, indeed, might warrant the creation of a new genus. The hesitancy to do so seems justified for the present because of the small number of species in the *Cytophaga* group and the present status of this field of research. Stanier's temporary groups might well be extended to three, including a fresh-water group. Whether or not *C. columnaris* is a true water form is of course not definitely known at the present time.

#### DIAGNOSIS

*Cytophaga columnaris* (Davis) Garnjobst; *pro synonym. Bacillus columnaris*  
Davis, 1922

**Morphology:** Highly flexible, singly occurring rods, 0.2 to 0.4 by 2.0 to 12.0 microns, capable of columnar, aflagellar, progressive movement on surfaces. Length very variable, average about 8.0 microns. In older cultures, cells from 12.0 to 20.0 microns are common. Branched cells are sometimes found. Gram-negative. Cells stain evenly by Giemsa's, Winogradsky's, and Feulgen's stains, and by Heidenhain's iron-alum hematoxylin. Thickened filaments and coiled (ring or coccoid) involution forms usually occur in old cultures. Star-shaped aggregates of cells form in liquid media and on agar with considerable moisture present.

**Peptone agar plate:** Growth begins as a pale yellow spreading swarm which rarely exceeds a diameter of 5.0 cm at any time. The branching, anastomosing columns of cells form a continually changing pattern, but gradually the pattern becomes fixed, beginning at the center, or older portion, and progressing outward. Warts and ring involution forms appear in the older portions. After 3 to 4 days, the color becomes distinctly yellow or old gold (sometimes with a tinge of orange) with a glistening surface.

**Non nutrient gelatin:** Rapid, stratiform liquefaction.

**Liquid media:** Growth is turbid and silky, sometimes with a pellicle which becomes yellow with age; at 13 to 18 C particularly, cells become piled up at the bottom of the tube.

**Utilizable carbon sources:** Proteinaceous materials are the only ones known. Cellulose, starch, and agar not attacked.

**Utilizable nitrogen sources:** Peptone and hydrolyzed casein are the only suitable ones known.

Hydrogen sulfide formed.

Catalase positive.

Indole not formed (Gneзда test).

**Salt concentration range:** To 0.3 per cent in 0.5 per cent tryptone solution.

Strictly aerobic.

**Optimum temperatures:** 25 to 30 C.

**Source:** Dermal lesions, fresh-water fishes.

**Habitat:** Cutaneous and connective tissues of fish.

## SUMMARY

*Bacillus columnaris* Davis, 1922, a dermal parasite of fresh-water fishes, has been isolated and grown successfully in peptone media and in hydrolyzed casein added to a mineral base. Its morphological characteristics, aflagellar type of creeping motility, and absence of microcysts in the life cycle place this organism in the order *Myxobacteriales*, family *Cytophagaceae*. Reasons for including *B. columnaris* in the genus *Cytophaga* are presented. This species was found to differ from other members of the genus in not attacking cellulose to any extent, in its parasitic nature, and in other minor respects.

I wish to express my thanks to Dr. H. S. Davis for helpful advice during the course of this work, and also for making the photomicrographs.

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# STUDIES ON THE ACTION OF SULFONAMIDES ON THE RESPIRATION AND GROWTH OF BACTERIA<sup>1</sup>

## B. FACTORS INFLUENCING THE CORRELATION OF THE INHIBITION OF RESPIRATION WITH THE INHIBITION OF GROWTH OF *ESCHERICHIA COLI*, *PNEUMOCOCCUS*, TYPE 1, AND *STAPHYLOCOCCUS AUREUS*

### I. THE INTERFERENCE OF THE EVOLUTION OF HYDROGEN WITH THE MEASUREMENT OF THE INHIBITION OF OXYGEN CONSUMPTION IN *ESCHERICHIA COLI*

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It is known that during aerobic and anaerobic oxidation of glucose by *Escherichia coli*, the reaction products include carbon dioxide and hydrogen. Equal amounts of these gases result from the enzymic decomposition of formate, which is an oxidation product of glucose. In addition, carbon dioxide is also produced from various other sources, e.g., glucolysis and decarboxylation of pyruvate. The evolution of carbon dioxide, arising from reactions between the acids formed from glucolysis with sodium bicarbonate, usually added to the glucolytic system, can largely be eliminated by omitting the addition of the carbonate. The gases formed under such conditions should principally consist of (a) carbon dioxide, resulting from the decarboxylation of pyruvate, etc., and (b) carbon dioxide and hydrogen resulting from the decomposition of formate. The presence of potassium hydroxide in the inner cup of the Warburg flask eliminates carbon dioxide from the system by absorption. This leaves hydrogen alone in the system, and hydrogen can be measured manometrically. Under these conditions, in the presence of sulfonamides manometric measurements will roughly show the degree of inhibition of hydrogen evolution, or the inhibition of the oxidation of glucose to formate. A parallel measurement with pure formate as substrate will, likewise, show whether or not its decomposition is inhibited by sulfonamides.

Under aerobic conditions, manometric measurements of the oxidation of glucose are complicated by the fact that oxygen is consumed, creating a negative pressure, and carbon dioxide and hydrogen are evolved, creating a positive pressure. Carbon dioxide can be eliminated by potassium hydroxide as described above. Under these conditions, the change in the pressure of the system is controlled by the balance of the volume of oxygen consumed and that of hydrogen evolved. Only a careful evaluation of these factors can enable us to show whether or not sulfonamides inhibit the oxidation of glucose by *E. coli* under these conditions.

<sup>1</sup> This investigation has been aided by a grant from The Josiah Macy, Jr., Foundation.

## RESULTS

*A comparison of the evolution of gases formed during the metabolism of sodium formate and glucose under anaerobic conditions. In the absence of added sodium bicarbonate, the evolution of gases from the metabolism of sodium formate and glucose was measured at hourly intervals for a period of 5 hours. These measurements were carried out in (a) M/150 phosphate of pH 7.2, and (b) M/150 phos-*

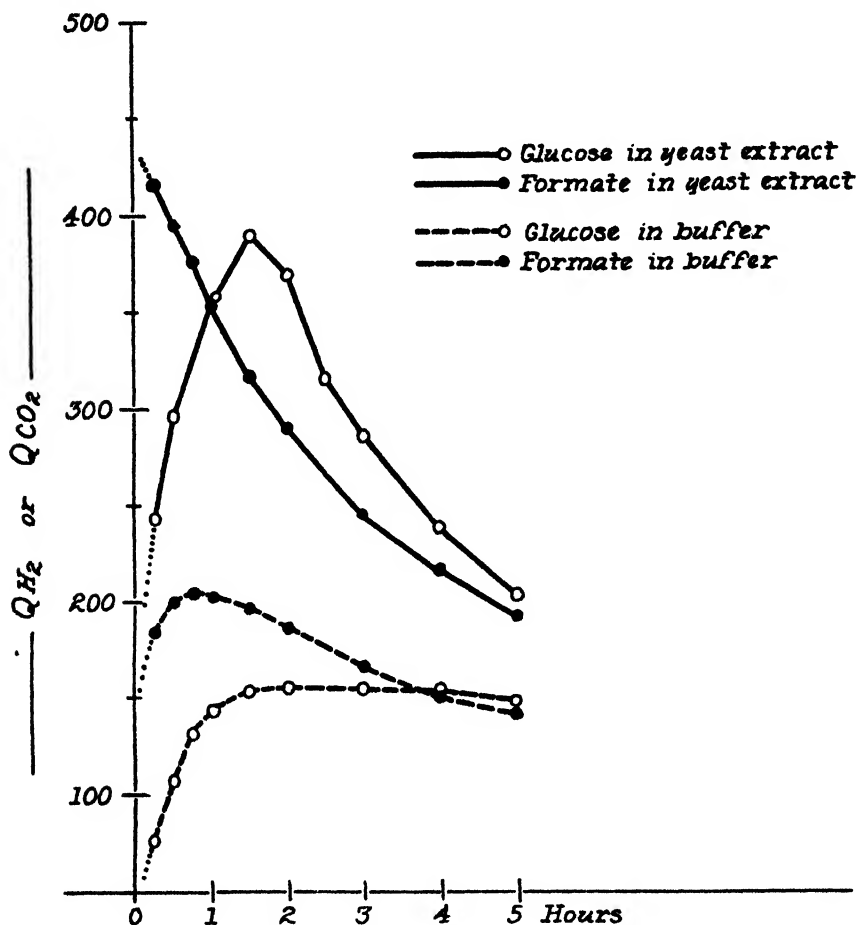


FIG. 1

phate containing a 0.05 per cent yeast extract. The inner cup of the Warburg flask did not contain potassium hydroxide. The volume of gas evolved in the sodium formate system represented, therefore, an equimolecular mixture of carbon dioxide and hydrogen. One half of the volume of gases evolved was used to calculate  $Q_{CO_2}$  or  $Q_{H_2}$ . The gases evolved in the system containing glucose were treated in the same manner for a comparison with sodium formate. Figure 1 represents the  $Q_{H_2}$  or  $Q_{CO_2}$  values plotted against time. It can be seen that

the curves are similar in shape.  $QH_2$  values sharply rose at the beginning, the peaks of optimal activities were reached within 30 to 90 minutes. Then they declined until the curves nearly flattened out. This is probably not due to the exhaustion of the substrates, but to the attainment of the possible reaction equilibrium:  $HCOOH \rightleftharpoons H_2 + CO_2$ .

The selective inhibition by sulfanilamide and *p*-aminobenzoic acid of the evolution of gases from glucose, and failure to inhibit the decomposition of formate by *E. coli*. The question to be answered is whether or not sulfonamides inhibit the decomposition of formate, or its formation from glucose. The results given in table 1 deal with this question. The initial weight of *E. coli* added to each anaerobic system was 1.5 mg. Measured at the end of a 5-hour period, there was no increase in the number of organisms in the systems containing sodium formate

TABLE 1

The selective inhibition of the evolution of hydrogen and carbon dioxide during the anaerobic metabolism of glucose, and failure to inhibit the evolution of these gases from formate by *Escherichia coli* by sulfanilamide and *p*-aminobenzoic acid

PERIOD	INHIBITION BY SULFANILAMIDE (0.04 M)				INHIBITION BY <i>p</i> -AMINOBENZOIC ACID (0.04 M)			
	Sodium formate		Glucose		Sodium formate		Glucose	
	Buffer	Yeast extract	Buffer	Yeast extract	Buffer	Yeast extract	Buffer	Yeast extract
hour	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
1	6	9	33	37	-9	12	28	50
2	11	4	36	35	-6	8	33	48
3	10	6	37	36	-5	6	36	36
4	10	6	38	39	-4	5	38	27
5	11	4	42	36	-1	3	39	8

Reaction system consisted of 5.8 ml of 0.85 per cent sodium chloride containing  $m/150$  phosphate of pH 7.2 and 18 mg of glucose, or 15 mg of sodium formate. The concentration of yeast extract was 0.05 per cent.  $T = 37.5^\circ C$ . Measurements were carried out in an atmosphere of 95 per cent  $N + 5$  per cent carbon dioxide with the Barcroft-Warburg setup.

with or without 0.05 per cent yeast extract, and in the system containing glucose without yeast extract. In systems containing both glucose and yeast extract, the weight of *E. coli* for the control was 2.25 mg, a 50 per cent increase in growth. In systems containing sulfanilamide and *p*-aminobenzoic acid, the weights of *E. coli* were, respectively, 1.873 mg and 2.063 mg. These correspond, respectively, to 50 and 25 per cent inhibition of growth. It is to be noted, as discussed below, that sulfanilamide under these conditions caused a 36 per cent inhibition of anaerobic respiration. In contrast, the metabolism of sodium formate, which did not support growth, was not significantly inhibited by sulfanilamide and *p*-aminobenzoic acid.

Hourly results of manometric measurements showed that the decomposition of sodium formate by *E. coli*, into hydrogen and carbon dioxide, was not inhibited by 0.04 *M* sulfanilamide and *p*-aminobenzoic acid. In contrast, the evolution of these gases during the anaerobic metabolism of glucose was inhibited



from 33 to 42 per cent (in buffer), and 35 to 39 per cent (in yeast extract) by sulfanilamide. Under identical conditions, the inhibition (in buffer) by *p*-aminobenzoic acid was from 28 to 39 per cent. However, in the presence of yeast extract, the initial 50 per cent inhibition by *p*-aminobenzoic acid gradually declined, and at the end of the 5-hour period only an 8 per cent inhibition was observed.

The above observations show that sulfanilamide inhibited the oxidation of glucose to formate, and not the decomposition of formate into carbon dioxide and hydrogen.

*Factors contributing to the evolution of hydrogen and carbon dioxide during the aerobic metabolism of sodium formate and glucose by Escherichia coli.* During the

TABLE 2

*Factors contributing to the evolution of hydrogen (and carbon dioxide) during the aerobic metabolism of sodium formate and glucose by Escherichia coli*

E. COLI	SODIUM FORMATE				GLUCOSE			
	Buffer	Yeast extract	Horse serum	Yeast extract + horse serum	Buffer	Yeast extract	Horse serum	Yeast extract + horse serum
<i>E. coli</i> in mg (3½ hr) ...	1.575	1.575	1.575	1.575	1.575	3.000	3.825	3.825
mm <sup>3</sup> H <sub>2</sub> liberated (3½ hr) .	122	623	404	497	724	1657	2222	2001
QH <sub>2</sub> . . . . .	23	114	75	90	133	207	235	212

*Reaction systems:* see footnote to table 1. Measurements were carried out in an atmosphere of air-oxygen. Carbon dioxide evolved during the reactions was absorbed in potassium hydroxide placed in the inner cup of the Warburg flask. Initial weight of *E. coli* added to each system was 1.575 mg. The concentrations of yeast extract and horse serum were, respectively, 0.05 and 25 per cent. Molarity of glucose and sodium formate were, respectively, 0.016 and 0.036.

The numerical values correspond to the positive pressure (hydrogen evolution) in excess of the negative pressure (oxygen consumption) exercised on the system. The observed pressures were converted into mm<sup>3</sup> values. The QH<sub>2</sub> values represent only a fraction of the total volume of hydrogen (and of carbon dioxide) produced.

aerobic metabolism of formate by *E. coli*, two simultaneous reactions take place: consumption of oxygen, yielding water and carbon dioxide; and decomposition of formate, yielding hydrogen and carbon dioxide. The aerobic metabolism of glucose by *E. coli* involves, therefore, consumption of oxygen with the simultaneous liberation of carbon dioxide and hydrogen. The determination of the degree of gaseous exchange in these systems involves technical difficulties which have not been overcome. With the elimination of carbon dioxide (absorption in KOH), the change in the pressure of the systems can serve as an indication of the preponderance of the volume of oxygen consumed, or that of hydrogen evolved. When the manometer does not register a change, it is an indication that the volume of oxygen consumed is equal to the volume of hydrogen evolved.

The numerical values, corresponding to the results of four different sets of conditions, presented in table 2, represent the volume of hydrogen evolved

(positive pressure) in excess of the unknown volume of oxygen consumed (negative pressure). It is clear that in these experiments the volume of hydrogen (and carbon dioxide) evolved markedly exceeded the volume of oxygen consumed. We are, therefore, left with no alternative but to determine the inhibition of the evolution of hydrogen by sulfonamides.

The results show that the presence of yeast extract in the systems caused a fivefold increase in the evolution of hydrogen from formate when compared with the results obtained in a simple buffer system. In the presence of horse serum, a similar effect was observed. With glucose the effect of yeast extract and horse serum was marked, but not so great.

TABLE 3

*The effect of the inhibition by sulfanilamide and p-aminobenzoic acid of the evolution of hydrogen during the metabolism of glucose on the measurement of the volume of oxygen consumed by E. coli*

E. COLI	YEAST EXTRACT			HORSE SERUM + YEAST EXTRACT		
	Control	Sulfanilamide (0.04 M)	p-Aminobenzoic acid (0.04 M)	Control	Sulfanilamide (0.04 M)	p-Aminobenzoic acid (0.04 M)
<b>A</b>						
<i>E. coli</i> in mg (3½ hr) . . .	3.900*	3.225	3.375	5.400	4.200	4.425
mm <sup>3</sup> O <sub>2</sub> consumed (3½ hr) .	1537	1530	1530	571	1679	1731
QO <sub>2</sub> . . . . .	158	179	174	46	164	163
Inhibition (%) . . . . .		-13†	-10		-256	-254
<b>B</b>						
<i>E. coli</i> in mg (3½ hr) . . .	3.525‡	3.000	2.663	3.525	3.750	3.825
mm <sup>3</sup> O <sub>2</sub> consumed (3½ hr) .	1309	1317	1283	0§	1410	683
QO <sub>2</sub> . . . . .	152	171	181	0	157	75
Inhibition (%) . . . . .		-12	-19		> -100	> -100

\* Initial weight of *E. coli* added to each system was 1.65 mg; yeast extract, 0.05 per cent; horse serum, 22 per cent.

† - = increase.

‡ Initial weight of *E. coli* added to each system was 1.388 mg.

§ 0 value means that the volume of oxygen consumed is equal to the volume of hydrogen evolved.

*The effect of the inhibition by sulfanilamide and p-aminobenzoic acid of the evolution of hydrogen during the oxidation of glucose on the measurement of the volume of oxygen consumed by Escherichia coli.* As shown before, during the aerobic oxidation of glucose by *E. coli*, in systems containing yeast extract, or serum and yeast extract, the volume of hydrogen evolved exceeded the volume of oxygen consumed. The results presented in table 3; on the other hand, show that the reverse may also be true. Considering this fact, and also the fact that sulfanilamide and p-aminobenzoic acid inhibited the evolution of hydrogen during the anaerobic metabolism of glucose, we would expect an apparent increase in the measurement of the volume of oxygen consumed in the presence of these

inhibitors over that consumed in their absence. That is, they partly remove, by inhibiting the evolution of hydrogen, the shield that obstructed a more complete measurement of the volume of oxygen consumed. The results presented in table 3 show that in systems containing yeast extract the effect of these inhibitors on the measurement of oxygen uptake underwent an increase of from 10 to 13 and from 12 to 19 per cent only. On the other hand, in the presence of horse serum and yeast extract, these inhibitors caused an increase of from 100 to 255 per cent in the measurement of the volume of oxygen consumed. As stated, this indicates a marked suppression of the evolution of hydrogen by these inhibitors.

These observations do not, in any way, give us a clue to the degree of the inhibition of oxygen consumed. We must, however, not overlook the possibility of the inhibition of oxygen consumption, despite the apparent increase in the measurement of oxygen uptake under these conditions.

TABLE 4

*Inhibition by sulfanilamide (0.04 M) of the aerobic respiration and growth of E. coli in the presence of glucose and neopeptone*

E. COLI	BUFFER		NEOPEPTONE (0.18%)		NEOPEPTONE (0.45%)	
	Control	Sulfanilamide	Control	Sulfanilamide	Control	Sulfanilamide
<i>E. coli</i> in mg (3 hr) . . . . .	1.350*	1.350	3.338	2.362	4.725	3.338
mm <sup>3</sup> O <sub>2</sub> consumed (3 hr) . . . . .	383†	305	1188	512	1352	778
QO <sub>2</sub> . . . . .	94	75	169	92	148	111
Inhibition of O <sub>2</sub> uptake (%) . . . . .		20		46		25
Inhibition of growth (%) . . . . .				29		29

\* Initial weight of *E. coli* added to each system was 1.350 mg.

† Carbon dioxide evolved during the reaction was absorbed in potassium hydroxide placed in the inner cup of the Warburg flask.

*Inhibition of oxygen consumption and growth of E. coli by sulfanilamide.* The inhibition of oxygen uptake was readily demonstrated in a system containing glucose and buffer, or glucose and neopeptone. The results (table 4) show that, in 0.18 per cent neopeptone, a 46 per cent inhibition of oxygen uptake was associated with 29 per cent inhibition of growth. In 0.45 per cent neopeptone, a 25 per cent inhibition of oxygen uptake was associated with 29 per cent inhibition of growth.

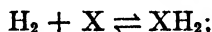
#### DISCUSSION

The decomposition of formate into equal volumes of hydrogen and carbon dioxide has been known for over sixty years (Hoppe-Seyler, 1876). Hoppe-Seyler stated that calcium formate on hydrolysis by bacteria yields calcium carbonate and equal volumes of carbon dioxide and hydrogen. More recently Quastel and Whetham (1925) have reported that there is a complete oxidation of formic acid (anaerobically, methylene blue technique) to carbon dioxide by *E. coli*, that there is a great increase in pH during the anaerobic oxidation of

formic acid, and that carbon dioxide is released on treatment with acid. These observations suggest an equilibrium reaction of  $\text{HCOOH} \rightleftharpoons \text{H}_2 + \text{CO}_2$ .

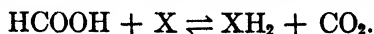
Stephenson and Stickland (1932) stated that there were three bacterial enzymes which were known to act on formic acid or hydrogen:

- (1) hydrogenase, which activates molecular hydrogen

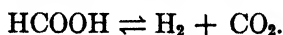


X = hydrogen acceptor (or carrier).

- (2) formic dehydrogenase which catalyses the reaction



- (3) formic hydrogenlyase, which catalyses the reaction



*E. coli* grown on plain broth has hydrogenase and formic dehydrogenase but no formic hydrogenlyase. Formic hydrogenlyase, in the case of *E. coli*, is stated to be an adaptive enzyme, i.e., it is formed only when the organism is grown in the presence of formate.

By measuring the equilibrium  $\text{H}_2 \rightleftharpoons 2\text{H}^+ + 2\epsilon$ , Green and Stickland (1934) found that *E. coli* and colloidal palladium are equivalent as catalysts. That is, an identical equilibrium point is obtained with both catalysts. The forward and backward reactions in  $\text{HCOOH} \rightleftharpoons \text{H}_2 + \text{CO}_2$ , according to Stephenson and Stickland (1932), is catalyzed by a single enzyme, hydrogenlyase. On the other hand, Ordal and Halvorson (1939) stated that the production of hydrogen and carbon dioxide from formic acid is the result of the combined action of the enzymes hydrogenase and formic dehydrogenase, which makes unnecessary the postulation of formic hydrogenlyase as an enzyme separate and distinct from hydrogenase and formic dehydrogenase.

In consideration of the reaction  $\text{HCOO}^- + \text{H}_2\text{O} \rightarrow \text{HCO}_3^- + 2\text{H}^+ + 2\epsilon$ , and  $2\text{H}^+ + 2\epsilon \rightarrow \text{H}_2$  (Borsook, 1935) and of other observations, Waring and Werkman (1944) suggest the possible hypothesis that the formation of molecular hydrogen from formate by certain bacteria involves the combined action of formic dehydrogenase, an intermediary electron mediator (probably cytochrome-like), and hydrogenase.

Considering the reaction  $\text{HCOOH} \rightleftharpoons \text{H}_2 + \text{CO}_2$  from the point of view of the kinetics of catalytic reactions, a single enzyme could be responsible for the equilibrium reaction in either direction. The theory of catalysis states that a catalyst does not alter the point of equilibrium but accelerates the rate at which the equilibrium state is attained. It accelerates the rate of the reverse reaction to the same extent as that of the forward reaction. These considerations make the postulation for the existence of two enzymes, exercising "direction specificity" in an equilibrium reaction, unnecessary. This appears to have been experimentally demonstrated by the findings of Green and Stickland (1934). In a study involving calculations of the free energy and heat of reactions, determination of equilibrium points, the specific inhibition of the enzyme system, etc.,

Woods (1936) concluded that hydrogenlyase catalyzes both the synthesis and the breakdown of formic acid. Kalnitsky, Wood, and Werkman (1943) also found that in the absence of hydrogenlyase, the reduction of  $\text{CO}_2$  from the medium by a cell-free enzyme preparation of *E. coli* is not the mechanism of formic acid formation. In view of these considerations, the statement of Stephenson and Stickland (1932) that  $\text{HCOOH} \rightleftharpoons \text{H}_2 + \text{CO}_2$  is catalyzed by a single enzyme hydrogenlyase appears to possess a theoretical as well as an experimental basis.

Ordal and Halvorson (1939) stated that the hydrogen produced from glucose by *E. coli* comes from formic acid, which is an intermediate product of the metabolism of glucose. The results of our experiments, presented in figure 1, seem to lend support to this view. This was further indicated by our finding that sulfanilamide was capable of inhibiting the production of hydrogen from glucose. In contrast, sulfanilamide (0.04 M) was incapable of inhibiting the production of hydrogen from formate. If there is in *E. coli* a single enzyme responsible for the liberation of hydrogen from both glucose and formic acid, it is reasonable to conclude that sulfanilamide should exercise a similar inhibitory effect on this single enzyme system. Absence of inhibition of the formic acid enzyme (hydrogenlyase, or dehydrogenase, or hydrogenase) and the presence of inhibition of the reactions producing hydrogen from glucose mean that sulfanilamide inhibits the respiratory enzyme system which oxidizes glucose to formic acid, and has no action on the metabolism of the latter. This means that there are two enzymes in *E. coli*. Waring and Werkman (1944) are of the opinion that the formic acid enzyme system contains an intermediate electron mediator which contains iron, probably in a manner similar to cytochrome. If we take for granted the existence of this electron mediator, we can readily see from the observations discussed that sulfanilamide does not exercise inhibitory action on the iron-containing component of the enzyme system. Bucca (1943) reported that sulfanilamide fails to inhibit catalase and peroxidase, but exercised marked inhibitory effect on the indophenol oxidase (cytochrome-c-cytochrome-oxidase) of gonococcus. In view of this observation, the presence of an iron-containing, cytochromelike electron mediator in *E. coli* would seem to be subject to question. However, since iron-porphyrin-containing enzymes, catalase or peroxidase, of the gonococcus are resistant to sulfanilamide, the question of the specificity of the protein components of enzymes in each species of bacteria must be assumed to play a significant role in this respect. This would explain also the selective resistance of formic acid dehydrogenase in *E. coli* to sulfanilamide. These considerations permit us to conclude that the inhibition of the oxidation of glucose (aerobically and anaerobically) is the result of the inhibition of an *E. coli* dehydrogenase, or flavoprotein, or both. This is in accordance with the "inhibition of respiration theory" (Sevag and Shelburne, 1942).

Ordal and Halvorson (1939), after reviewing various reports concerning the ability of *E. coli* to produce hydrogen from glucose and formate, are of the opinion that the enzyme can be permanently or temporarily lost. They found that suspensions of *E. coli* grown in glucose or maltose broth produced hydrogen from formate, glucose, or maltose at a rapid rate. In our experiments, *E. coli* was

grown for 18 hours in phosphate broth of pH 7.4 containing 0.5 per cent glucose. The results plotted in figure 1 show that washed suspensions of *E. coli* exhibit moderate activity in producing hydrogen from both formate and glucose in a buffer mixture. On the other hand, in the presence of 0.05 per cent yeast extract the activity was from 2- to 3-fold greater. This indicates that coenzyme factors, present in yeast extract, play an important role in the activity of cell suspensions. This is also evident from the results presented in table 3 (Section A). An analysis of the results show that, in yeast extract, the  $QO_2$  was 158, which is interpreted as showing that the volume of oxygen consumed exceeded the volume of hydrogen evolved by  $QO_2$  158. In the presence of horse serum and yeast extract  $QO_2$  was 46, which means that a greater volume  $(158 - 46/158 \times 100 = 71 \text{ per cent})$  of hydrogen was produced than in the yeast extract medium. Evidently the factors present in serum are much more effective than those present in yeast extract alone. It must be remembered that these activations are obtained without previous training, or adaptation, of *E. coli* in these media. A similar analysis of the results presented in table 3 (Section B) leads to the same conclusion. In yeast extract  $QO_2$  was 152, and in horse serum and yeast extract  $QO_2$  was 0. This means that when serum and yeast extract are used the volume of hydrogen evolved is equal to that of oxygen consumed; in other words, a much greater volume of hydrogen was evolved in yeast extract and serum than in yeast extract alone.

In the presence of 0.04 M sulfanilamide the production of hydrogen during the metabolism of glucose and growth of *E. coli* was inhibited by  $> 255$  per cent, which was shown by an increase, equal in degree, in the measurement of the volume of oxygen consumed. This increase resulted from the inhibition of the oxidation of glucose to formic acid.

Under the conditions described, yielding interfering amounts of hydrogen, the inhibition of oxygen consumption could not be determined. However, in experiments carried out in neopeptone, there was no evidence regarding the production of hydrogen; under these conditions the volume of oxygen consumed was marked, giving 94 for  $QO_2$ . Sulfanilamide exercised from 20 to 46 per cent inhibition of oxygen uptake and 29 per cent inhibition of growth.

The inhibition of both the evolution of hydrogen and consumption of oxygen, and of growth, shows that sulfanilamide inhibits the respiratory enzymes of *E. coli*. Kohn and Harris (1941) reported that the oxygen consumption of suspensions of *E. coli* was not affected by sulfanilamide. However, a calculation of their results by Henry (1943) showed from 4 to 37 per cent inhibition in  $10^{-3}$  to  $10^{-4}$  M sulfanilamide.

#### SUMMARY

Hydrogen is produced during the aerobic and anaerobic metabolism of formic acid and glucose by *Escherichia coli*. The hydrogen produced from glucose appears to result from formic acid, which is formed from glucose as a reaction product.

Considering the reaction  $HCOOH \rightleftharpoons H_2 + CO_2$  from the standpoint of the

kinetics of catalytic reactions, there appears to be reason for believing that a single enzyme is responsible for the forward and reverse reaction. Postulation of the existence of two enzymes is not supported.

Sulfanilamide inhibited the oxidation of glucose to formic acid. It had no effect on the metabolism of formic acid. Sulfanilamide also inhibited the consumption of oxygen in the presence of glucose, and growth to a comparable degree.

These considerations show that sulfonamides inhibit the respiratory enzymes of *Escherichia coli*.

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# STUDIES ON THE ACTION OF SULFONAMIDES ON THE RESPIRATION AND GROWTH OF BACTERIA<sup>1</sup>

## B. FACTORS INFLUENCING THE CORRELATION OF THE INHIBITION OF RESPIRATION WITH THE INHIBITION OF GROWTH OF *ESCHERICHIA COLI*, *PNEUMOCOCCUS*, TYPE I, AND *STAPHYLOCOCCUS AUREUS*

## II. EFFECT OF SERUM ON THE INHIBITION OF RESPIRATION AND GROWTH OF *PNEUMOCOCCUS*, TYPE I, AND *STAPHYLOCOCCUS AUREUS* BY SULFONAMIDES AND *p*-AMINO BENZOIC ACID

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In studies dealing with the mode of action of sulfonamides, one of the objectives is to obtain information regarding the action of drugs in the highly complex environment *in vivo*. It is likewise the objective of such studies to demonstrate why certain drugs are most effective against certain bacteria, and others are completely ineffective. To correlate the results of *in vitro* studies with those obtained in *in vivo* experiments, it is necessary that at least some of the components of environments *in vivo* be incorporated in the experiments *in vitro*. In view of these considerations, in our earliest studies (Sevag and Shelburne, 1942a, 1942b), the correlation of the inhibition of respiratory enzymes with the inhibition of growth by sulfanilamide was made in media containing serum and yeast extract. The results showed that inhibition of respiration of *Streptococcus pyogenes* resulted in proportional inhibition of growth. In contradiction of the results obtained by us, Wyss, Strandkov, and Schmelkes (1942) reported that inhibition of respiration studies did not show true sulfanilamide activity. Since these investigators experimented in simple buffer media, their conclusion may no doubt be accurate as far as the conditions they used are concerned. Their results, however, neither resemble the results obtained under the conditions of our experiments, nor can they serve to bring out true sulfonamide activity. As will be shown in the present study of the factors controlling sulfonamide activity, serum proteins play a very important role. Most investigators who have confined themselves to the study of the action of sulfonamides on bacteria in restricted simple media (synthetic or casein hydrolyzate), appear to have failed to take into consideration the importance of serum proteins. Although experiments carried out in simple media may yield valuable information as to the effect of various substances added to the medium, etc., they fail to lend themselves to a satisfactory correlation with clinical observations. For this and similar reasons, theories formulated for the correlation of certain physico-chemical properties of

<sup>1</sup> This investigation has been aided by a grant from The Josiah Macy, Jr., Foundation.



sulfonamides with their action on bacteria in simple media fail to answer adequately the question of the mode of action of sulfonamides.

With consideration of the problems briefly discussed, we have carried out comparative experiments in systems with and without serum proteins. Our findings should be of clinical significance.

*Inhibition by para-, ortho-, and meta-aminobenzenesulfonamides, and p-aminobenzoic acid of the respiration and growth of pneumococcus type I, in simple medium, and absence of inhibition in serum.* The results presented in table 1 show that in yeast extract *para*-, *ortho*-, and *meta*-aminobenzenesulfonamides, and *p*-aminobenzoic acid inhibited the respiration, respectively, 38, 77, 59, and 27 per cent. In the presence of 27 per cent horse serum, these inhibitions were, respectively, -37, -49, -33, and -24 per cent. That is, the inhibitions were not only abolished, but respiration was increased by these drugs. Similarly, in the presence of yeast extract these drugs inhibited the growth, respectively, 45, 70, 64, and 32 per cent. In the presence of serum these inhibitions were reduced, respectively, to 17, 35, 8, and -6 per cent. These observations show that serum proteins are critical factors in the degree of inhibition exercised by sulfonamides. They likewise show that bacterial enzyme proteins compete with serum proteins for these drugs. This aids in explaining why sulfanilamide is not an effective drug against pneumococcal infections.

*Failure of 0.04 M para-, meta-, ortho-aminobenzenesulfonamides, and p-aminobenzoic acid to inhibit the respiration and growth of Staphylococcus aureus.* It is known that sulfanilamide and, in fact, most of the sulfonamides are of very little use in the treatment of infections with *Staphylococcus aureus*. It is therefore of interest to learn to what extent clinical observations can be correlated with the results of respiration experiments under conditions somewhat suggestive of conditions *in vivo*. Inhibition of respiration and growth was measured under the following conditions: (a) a mixture of phosphate buffer and glucose, to determine the effect of sulfonamides on the respiration of *resting* cells; (b) a mixture of buffer, glucose, and 0.3 per cent yeast extract (table 2), to determine the effect of the drug on respiration and growth simultaneously in the absence of serum proteins; and (c) a mixture of buffer, glucose, yeast extract, and 50 per cent serum (table 3). The results of these experiments are presented in tables 2 and 3.

It can be seen (table 2) that 0.04 M solution of *para*-, *meta*-, *ortho*-aminobenzenesulfonamides, and *p*-aminobenzoic acid do not exercise any appreciable degree of inhibition on the aerobic respiration and growth (5-hour period) of a laboratory staphylococcal strain (P78) under the first two conditions. It must be mentioned here that yeast extract, which contains practically all the vitamins and other accessory substances present in the yeast cell, is a much more complex medium than casein hydrolyzate or synthetic medium. The *p*-aminobenzoic content of the yeast extract was too small to be detected by the method of Bratton and Marshall (1939) by which 0.02  $\mu$ g per ml volume could easily be measured. Because of the presence of these accessory factors, bacteria are much more resistant to the action of sulfonamides in yeast extract than in casein hydrolyzate or synthetic medium. This will be further discussed.

TABLE 1  
Inhibition of the aerobic respiration and growth of *pneumococcus* by 0.04 M para-, ortho-, and meta-aminobenzenesulfonamides, and para-aminobenzoic acid (p-ABA) in simple medium, and absence of inhibition in serum (97 per cent)

PNEUMOCOCCUS, TYPE 1*	CONTROL		para		ortho		meta		p-ABA	
	Yeast extract	Yeast extract + serum	Yeast extract	Yeast extract + serum	Yeast extract	Yeast extract + serum	Yeast extract	Yeast extract + serum	Yeast extract	Yeast extract + serum
Pneumococci in mg (5 hr) . . . . .	1.333	1.66	0.869	1.435	0.615	1.189	0.677	1.558	1.005	1.74
mm <sup>3</sup> oxygen consumed (5 hr) . . . . .	1181	1052	473	1290	124	1125	247	1320	654	1370
QO <sub>2</sub> . . . . .	177	127	109	174	40	189	73	169	130	158
Inhibition of O <sub>2</sub> uptake (%) . . . . .			38†	-37	77	-49	59	-33	27	-24
Inhibition of growth (%) . . . . .			45	17	70	35	64	8	32	-6

\* Weight of pneumococci used as inoculum was 0.615 mg.

† Numbers without any sign indicate inhibition, the figures with — sign indicate per cent increase over that of control.

*Reaction System:* with or without horse serum, consisted of sterile solutions containing 3 mg of yeast extract + 18 mg of glucose + 0.05 ml of water-clear catalase solution in 5.5 ml of M/30 phosphate buffer of pH 7.4. In these experiments glucose was added last to the system after equilibration of temperature at 37.5 C, etc. At the end of the experiment, the purity of the cultures in each system was established by microscopic examination. Weight of bacteria was determined turbidimetrically by means of the Klett-Summerson photoelectric colorimeter, standardized against pneumococcal nitrogen.

TABLE 2  
Comparison of the inhibitory effects of 0.04 M of para-, meta-, and ortho-aminobenzenesulfonamides and para-aminobenzoic acid on the aerobic respiration and growth of *Staphylococcus aureus* (P78)

STAPHYLOCOCCUS AUREUS (P 78)	CONTROL		AMINO BENZENESULFONAMIDES							
			para		meta		ortho		p-ABA	
	Buffer	Yeast extract	Buffer	Yeast extract	Buffer	Yeast extract	Buffer	Yeast extract	Buffer	Yeast extract
Staphylococci in mg (5 hr).....	0.65*	3.218	0.65	3.185	0.65	3.250	0.65	2.795	0.65	3.283
Mm <sup>3</sup> O <sub>2</sub> consumed (5 hr).....	272	2256	232	1932	208	1868	295	2040	332	2401
QO <sub>2</sub> .....	84	238	71	201	64	192	91	237	102	244
Inhibition of O <sub>2</sub> uptake (%).....			14	14	23	18	-10	-2	-23	-5
Inhibition of growth (%).....				1		1		13		-2

\* The staphylococcal suspension used in these experiments was made from an 18-hour culture in extract broth (glucose absent) seeded from a culture on extract agar.

Reaction Systems: (a) Buffer = 5.1 ml of M/30 phosphate of pH 7.4 + 0.2 ml of glucose solution (25 mg) + 0.2 ml of staphylococcal suspension (0.65 mg); (b) Yeast extract medium = same as in (a) except that the phosphate buffer contained 15 mg of yeast extract per flask.

The sign — indicates an increase over that of control values.

In another experiment (table 3) a strain which had been rendered resistant to sulfonamides was used. Measurements were made at intervals of one and two hours for a period of 10 hours. It was hoped to find whether or not there was any inhibition of respiration and growth at the very beginning. It can be seen that during the first 1-hour period the weight of cocci was increased from 2- to 3-fold (anaerobic and aerobic). At the end of the 10-hour period it had increased from 6- to 10-fold. Sulfanilamide exercised no inhibitory effect on the respiration and growth throughout the whole period. A measurable degree of inhibition, observed under anaerobic conditions, was practically abolished toward the end of the 10-hour period.

TABLE 3

*Comparison of the hourly effect of sulfanilamide (SA) on the aerobic and anaerobic respiration and growth of Staphylococcus aureus resistant to sulfonamides*

AEROBIC RESPIRATION AND GROWTH					ANAEROBIC RESPIRATION AND GROWTH				
Period	Wt. of staphylococci: control	QO <sub>2</sub> control	Inhibition by 0.04 M SA		Period	Wt. of staphylococci: control	QCO <sub>2</sub> control	Inhibition by 0.04 M SA	
			O <sub>2</sub>	Growth				CO <sub>2</sub>	Growth
hour	mg	mm <sup>3</sup>	per cent	per cent	hour	mg	mm <sup>3</sup>	per cent	per cent
0	0.39				0	0.293			
1	1.105	256	2	12	1	0.520	258	32	0
2	1.852	164	11	7	2	0.910	262	26	14
3	2.600	120	0	0	3	1.300	246	24	15
5	2.795	107	7	14	4	1.918	236	17	34(?)
7½	3.055	88	3	4	5	1.983	193	13	16
10	3.055	78	3	5	6	1.983	167	3	8

*Reaction Systems:* *Aerobic:* 2.6 ml of normal horse serum containing 2.5 mg of yeast extract + 10 mg of glucose and 0.2 ml of staphylococcal suspension in M/30 phosphate of pH 7.4. *Anaerobic:* Same as in aerobic experiments but containing 0.2 ml of 1 M NaHCO<sub>3</sub> in an atmosphere of 95 per cent N + 5 per cent CO<sub>2</sub>.

The staphylococcal suspension was prepared from a 16-hour extract broth (glucose absent) culture seeded with a pin-point inoculum from an 8-hour culture on extract agar (glucose absent).

If the production of *p*-aminobenzoic acid is responsible for the absence of inhibition, it is to be expected that there should be a decided difference between the inhibiting effects exercised by sulfanilamide at the beginning and at the end of the 10-hour period, for the amount of *p*-aminobenzoic acid, produced during growth, will be markedly smaller during the initial than during the final period. The production of *p*-aminobenzoic acid, for which there is as yet no rigid evidence, could not therefore account for the resistance of staphylococci to sulfonamides under these conditions.

*Inhibition by sulfanilamide and sulfathiazole of the growth of ten strains of staphylococcus in vitamin-free casein hydrolyzate, and absence of inhibition in 50 per cent serum medium.* (Experimental results from a study by Sevag and Green, 1944a, 1944b.) The results presented in table 4 show that the growth of all nine

strains of *Staphylococcus aureus* in vitamin-free casein hydrolyzate (SMACO) is inhibited. In the presence of serum proteins these inhibitions are either abolished or reduced to a negligible degree. The results of pH measurements, at the end of the growth period, show that slight variations in pH cannot account for the absence or presence of the inhibition of growth in these media. In correlating these results with the inefficacy of sulfonamides in clinical tests, only the results obtained in experiments with serum are of significance.

TABLE 4

*Inhibition by sulfonamides of the growth in casein hydrolyzate (vitamin-free) and absence of inhibition of the growth in serum-glucose mixture of the various strains of Staphylococcus (24-hour period)*

STRAINS OF <i>S. AUREUS</i>	CASEIN HYDROLYZATE, 1 PER CENT						HORSE SERUM, 50 PER CENT		
	Without glucose			Glucose, 0.5 per cent			Glucose, 0.5 per cent		
	Control	% Inhibition by		Control	% Inhibition by		Control	% Inhibition by	
	Growth	SA 0.04 M	ST 0.0066 M	Growth	SA 0.04 M	ST 0.0066 M	Growth	SA 0.04 M	ST 0.0066 M
Resistant (R. Finland).....	73*	28	31	96	0	31	65	0	15
Susceptible (S. Finland).....	71	35	42	86	16	41	56	5	3
Rose.....	87	43	43	91	26	44	62	3	8
P. G. H. ....	54	18	6	81	50	53	65	31	1
P 78.....	66	40	40	98	45	53	60	16	15
B-515.....	42	24	36	86	23	57	52	20	15
B-515a.....	54	9	15	123	46	49	64	17	15
B-523.....	44	46	46	28	57	50	61	7	9
B-523a.....	67	20	21	112	27	37	68	11	11
<i>S. albus</i> .....	29	13	13	29	10	31	36	15	3
pH of the culture fluid after 24 hr growth†.....	7.33-7.90	7.30-7.58	7.18-7.42	4.90-6.69	5.68-7.01	6.0-6.92	6.0-6.40	6.14-6.56	6.28-6.52

\* Readings in growth column represent photoelectric colorimetric (Klett-Summerson) turbidities obtained with 3 ml culture diluted to 10 ml in a standard tube.

† All 10 strains of *Staphylococcus* showed the same pH under the above conditions.

#### DISCUSSION

It is known that sulfanilamide is an effective chemotherapeutic agent against *Streptococcus pyogenes*. In contrast, it is comparatively ineffective against infections with pneumococcus and *Staphylococcus aureus*. It is also known that in contrast to *para*-aminobenzenesulfonamide (sulfanilamide), *meta*- and *ortho*-aminobenzenesulfonamides are chemotherapeutically ineffective. In *in vitro* studies, dealing with the mode of action of sulfonamides in relation to their efficacy in infectious diseases, the results must account for these differences. This necessitates the consideration of the role of some of the components of the

*in vivo* environment on the action of drugs on bacteria. Of these components, the effects of serum proteins, metabolites, protein degradation products, and vitamins and coenzymes must play important roles. In synthetic media, or in simple casein hydrolyzate, these factors are absent. It would, therefore, be expected that the infectious agents should be more susceptible to the action of the drugs in simple media than in media containing serum fractions, or vitamins, or both.

A previous study (Sevag and Shelburne, 1942a) concerning the inhibitory effect of sulfanilamide on respiration and growth in a mixture of yeast extract and whole serum showed that inhibition of respiration resulted in a proportional inhibition of growth. On the basis of this and similar studies the authors called attention to the fact that sulfonamide action involves blocking of bacterial respiratory enzymes. The results with streptococci correlate with the clinical action of the drug. The question arises as to why the same drug is ineffective against pneumococci and staphylococci. The results show that, in the absence of serum, but in the presence of yeast extract, *para*-, *meta*-, *ortho*-aminobenzenesulfonamides, and *p*-aminobenzoic acid exercised strong inhibitions on pneumococci. In contrast, in the presence of 27 per cent serum, the inhibitions were not only abolished, but there was an increase of from 24 to 49 per cent in respiration. These results are in harmony with clinical studies by which the inefficacy of these substances have been demonstrated.

The results of the present study also show that these drugs do not exercise any inhibitory effect on the respiration and growth of staphylococci in buffer, or yeast extract, or in yeast extract and serum. These results likewise are in agreement with clinical observations. The inability of these substances to inhibit the physiological activities of staphylococci is not due to the presence of *p*-aminobenzoic acid (Sevag and Green, 1944a). There is as yet no rigid evidence that these bacteria elaborate or require *p*-aminobenzoic acid. The results of an experiment involving hourly measurement of respiration and growth also showed no effect by sulfonamides. If the accumulation of *p*-aminobenzoic acid had been responsible for the absence of inhibition, it could have been expected that the drugs would have exercised a marked inhibition during the early hours of respiration and growth. No such effect was observed.

The results reported show that in the absence of inhibition of respiratory enzymes the drugs fail to inhibit growth. That is, the inhibition of respiratory enzymes can bring out the true sulfonamide activity, provided experiments are planned in the light of these considerations. Wyss, Strandkov, and Schmelkes (1942) in a study with certain strains of *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Escherichia coli* could not observe any differences between the degrees of inhibition exercised by *para*-, *meta*-, and *ortho*-aminobenzenesulfonamides. In this report, they likewise could not demonstrate a difference between sulfonamide-resistant and sulfonamide-susceptible strains. These investigators used a simple salt mixture containing glucose as the reaction system. The reason for this result is no doubt the failure to take into consideration certain basic factors which operate in more complex environments in which the specificities of the

drugs are tested. Our findings, which are more in agreement with clinical observations, are not in agreement with the conclusions of Wyss *et al.*

Our observations would appear to show that the affinity of the respiratory enzyme-proteins of staphylococci for sulfonamides is readily overcome by environments containing vitamins, coenzymes, and serum proteins. This is demonstrated by a comparative study of the effect of sulfanilamide and sulfathiazole on the growth of 10 strains of staphylococci in vitamin-free (SMACO) casein hydrolyzate, and in the medium containing dialyzed serum. The growth in casein hydrolyzate was markedly inhibited by both drugs, with the exception of the growth of *Staphylococcus albus*. In the presence of dialyzed serum, on the other hand, the inhibitions were either abolished or markedly reduced. These findings show that serum proteins combine with sulfonamides and thereby protect staphylococci. The competition exercised among the drugs, serum proteins, and bacterial enzymes are presented schematically in part A, article III, of this series of papers. These observations are in agreement with clinical results.

In addition to serum proteins, the metabolism of staphylococci in a medium containing riboflavin, pantothenic acid, and tryptophane plays an important role in antagonizing the complete inhibition of growth by sulfathiazole (Sevag and Green, 1944b).

In connection with this discussion and a comparison of the absence of inhibition of the oxygen consumption (table 2) with the inhibition of growth in casein hydrolyzate alone (table 4) by sulfonamides, one must also keep in mind the fact that, in experiments which attempt to demonstrate the relation of inhibition of respiratory enzymes and of growth, measurement of the inhibition of oxygen is not always adequate. After all, oxygen is one of numerous hydrogen acceptors functioning in the metabolism of bacteria. If the organism can utilize amino acids advantageously in a manner not involving the participation of oxygen as hydrogen acceptor, inhibition of the respiratory enzymes involved in the anaerobic metabolism must be measured by another suitable quantitative method.

#### SUMMARY

*Para*-, *meta*-, *ortho*-aminobenzenesulfonamides, and *para*-aminobenzoic acid inhibited the respiration and growth of pneumococcus type I, when measured in glucose and yeast extract medium. These inhibitions were either markedly reduced, or abolished, when the system contained serum proteins.

These substances did not inhibit either respiration or growth of staphylococcal strains in media containing (a) buffer mixture and glucose; (b) glucose and yeast extract; and (c) glucose and serum with or without yeast extract.

Sulfanilamide and sulfathiazole inhibited the growth of staphylococcal strains in vitamin-free casein hydrolyzate. These inhibitions were either abolished or markedly reduced when serum was also included in the medium.

These observations are in agreement with clinical results with respect to the inefficacy of the above-mentioned drugs. They indicate that serum proteins compete with bacterial enzymes for these drugs. In this respect, results of

studies on the inhibition of respiratory enzymes and growth by sulfonamides appear to be in agreement.

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# IN VITRO STUDIES ON THE ANTIBACTERIAL ACTIONS OF PARA-AMINOMETHYLBENZENESULFONAMIDE<sup>1</sup> DERIVATIVES<sup>2</sup>

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Early investigators apparently concluded on the basis of studies of a limited number of compounds that slight alterations or substitutions in the N<sup>4</sup> position of sulfanilamide would reduce or inactivate completely the effects of the compounds against bacteria, both *in vitro* and *in vivo*. Subsequent studies therefore involved almost entirely a consideration of compounds in which additions were made in the N<sup>1</sup> position of the molecule. Substitution in the latter position by heterocyclic groups has resulted in the preparation of many commonly known sulfonamides, such as sulfapyridine, sulfathiazole, and sulfadiazine.

Miller *et al.* (1940) synthesized and tested the chemotherapeutic value of a series of sulfonamide compounds of the general types,  $\text{H}_2\text{N}(\text{CH}_2)_m\text{---}\text{C}_6\text{H}_4\text{---}(\text{CH}_2)_n\text{---}\text{SO}_2\text{NH}_2$  and  $\text{H}_2\text{N}(\text{CH}_2)_x\text{SO}_2\text{NH}_2$ . On the basis of their findings, they concluded that separation of either the amino group or the sulfanilamide group from the benzene nucleus or the substitution of an aliphatic residue for the benzene nucleus in the sulfanilamide structure resulted in compounds which were of little significant value as chemotherapeutic agents in experimentally induced streptococcal infections in mice. Included among the various compounds tested by Miller and his co-workers were *p*-( $\beta$ -aminoethyl)-benzenesulfonamide and *p*-aminomethylbenzenesulfonamide.

Klarer (1941), working independently of this group, described also a series of sulfonamides, among which was *p*-aminomethylbenzenesulfonamide in which the 4-amino group is bound to the benzene nucleus through an aliphatic residue. He noted that while removal of the amino group from the benzene nucleus resulted in a lowering of activity against streptococcal infections, the activity increased against certain anaerobic bacteria. Therefore, the author concluded that the amino group bound to the nucleus formerly considered necessary for the action of sulfonamides can accordingly be replaced by an amino group in the side chain, whereby new compounds active specifically against anaerobic bacteria may be obtained.

Since 1941, *p*-aminomethylbenzenesulfonamide, in combination with prontosil and sulfanilamide (1:9 and 1:1 respectively), has been available commercially abroad. Beyer (1941) employed *p*-aminomethylbenzenesulfonamide-sulfanil-

<sup>1</sup> "Sulfamylon" is the trade name for *p*-aminomethylbenzenesulfonamide. The drug has also been known abroad as "marfanil" and "mesudin," and in this country as "homosulfanilamide."

<sup>2</sup> Presented in part at the 45th Annual Meeting of the Society of American Bacteriologists, New York City, May 3, 1944.

amide powders in a proportion of 1:9 in fresh wounds as a prophylactic and in infected wounds as an antiseptic, and found healing to progress with surprising rapidity. No harmful effects from use of the powder were observed. Bacteriological examination showed that *p*-aminomethylbenzenesulfonamide, applied to wounds infected with streptococci, resulted in a complete disappearance of the organisms within four days. On the basis of these findings, Beyer concluded that *p*-aminomethylbenzenesulfonamide is a valuable adjunct to surgical measures in the treatment of wounds.

In a series of investigations on the treatment of gas edema infections using sulfonamides, Domagk (1942) found *p*-aminomethylbenzenesulfonamide to be the most active compound studied. In experiments with pararauschbrand bacillus ("*Vibrio septique*") over 80 per cent of the animals treated with *p*-aminomethylbenzenesulfonamide survived as compared with 20 per cent of the animals treated with sulfanilamide and 35 per cent with uliron C (N<sup>4</sup> sulfanilyl sulfanilamide). The superiority of *p*-aminomethylbenzenesulfonamide over other sulfonamides was also demonstrated *in vitro*. Domagk, furthermore, described the use of the preparation in gaseous wound infections in man.

McKee *et al.* (1943), comparing the relative *in vitro* effects of several sulfonamides, noted that *p*-aminomethylbenzenesulfonamide was considerably more active than sulfanilamide, sulfathiazole, and sulfadiazine against a sulfonamide-fast strain of *Clostridium welchii*. These workers confirmed the early observation of Schreus (1942) that *p*-aminomethylbenzenesulfonamide is not inhibited by *p*-aminobenzoic acid.

Clark (1943) found that sulfanilamide and sulfathiazole were more active than *p*-aminomethylbenzenesulfonamide *in vitro* against *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Staphylococcus aureus*. However, high concentrations of the latter compound proved to be more effective than sulfathiazole against a sulfonamide-resistant strain of *Staphylococcus aureus*.

The significance of having a suitable drug for the local treatment of war-wound infections caused by the gas-gangrene group of anaerobic bacteria would appear at this time to merit further studies on derivatives of the compound mentioned. In the present communication is presented a series of *in vitro* studies on the action of several derivatives of *p*-aminomethylbenzenesulfonamide against a number of anaerobic bacteria, several of which are associated with gas-gangrene infections. Beta hemolytic streptococcus C-203, types I, II, and III pneumococci, a viridans streptococcus, *Streptococcus faecalis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were also studied. The compounds included the hydrochlorides of *p*-aminomethylbenzenesulfonamide, *p*-( $\alpha$ -aminoethyl)-benzenesulfonamide, and *p*-( $\beta$ -aminoethyl)-benzenesulfonamide; and the sulfosalicylate, mandelate, and methane-bis (2-hydroxy-3-naphthoate) salts of the drug. The antibacterial effects of these compounds were compared with those of sodium sulfathiazole and sodium sulfadiazine. Included in this report are the results of studies in which the various sulfonamides were tested in the presence of *p*-aminobenzoic acid and *p*-aminomethylbenzoic acid. The latter is an analogue of *p*-aminobenzoic acid which also contains the same *p*-aminomethyl group present in *p*-aminomethylbenzenesulfonamide.

I. *Effects of compounds against anaerobic bacteria.* Increasing dilutions of the compounds were made in Brewer's thioglycollate (anaerobic) medium (1940) to give drug concentrations of 1:100 down to and including 1:800. The compounds showing an antibacterial effect in this dilution range were tested further in serial dilutions of 1:1,000 up to and including 1:64,000. The drug-broth solutions were distributed in 10-ml amounts in cotton-plugged test tubes and sterilized by autoclaving at 10 pounds for 10 minutes. Upon cooling, to each series of drug-broth tubes and control medium lacking a drug was added one 4-mm loopful of an undiluted 24-hour thioglycollate medium culture of an anaerobe. The inoculated tubes were placed at 37 C to incubate and examined for the presence of visible growth at the end of 24, 48, and 72 hours. Lack of growth in the tubes at the end of 24 hours was taken as evidence of a bacteriostatic effect of the drug against the test organism. Tubes showing no growth at the end of 72 hours were tested for the presence of viable organisms by transferring three 4-mm loopfuls of the test solution to a tube of sterile thioglycollate medium. Failure of growth to appear in the subculture tube was assumed to indicate a bactericidal action on the part of the sulfonamide in the original test mixture.

The data on the antibacterial effects of the several *p*-aminomethylbenzene-sulfonamide derivatives, sodium sulfathiazole, and sodium sulfadiazine against certain anaerobic bacteria, which have been maintained in laboratory media for a number of years (from Dr. M. H. Soule), are presented in tables 1 and 2. It may be observed that, in general, sulfosalicylate, mandelate, and methanebis(2-hydroxy-3-naphthoate) salts of *p*-aminomethylbenzenesulfonamide are similar in activity to the hydrochloride in inhibiting and destroying the test organisms. Under like experimental conditions, sodium sulfathiazole and sodium sulfadiazine failed to show evidence of complete bacteriostasis at the end of 24 hours in the highest concentrations tested.

It is of some interest to note that although the *p*-( $\alpha$ -aminoethyl)-benzene-sulfonamide derivative showed evidence of an antibacterial action against the various organisms, this same effect was entirely lacking when the *p*-( $\beta$ -aminoethyl)-benzenesulfonamide analogue of *p*-aminomethylbenzenesulfonamide was used. This would indicate that removal of the amino group from the position adjacent to the benzene nucleus to the  $\beta$ -position in the ethyl residue results in a marked decrease or a complete absence of an antibacterial effect of the compound *in vitro*. The structural formulae of the two ethyl analogues of *p*-aminomethylbenzenesulfonamide are indicated as follows:

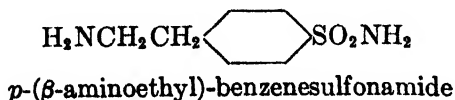
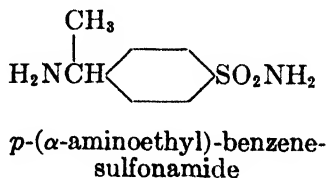
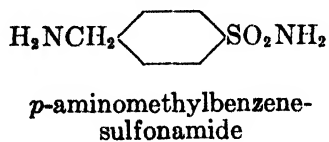


TABLE 1

*Highest dilution of compound showing antibacterial effect against several strains of anaerobic bacteria*

COMPOUND	C. WELCHII		C. HISTOLYTICUM		C. OEDEMATIENS		C. SEPTICUM	
	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc
<i>p</i> -Aminomethyl-benzenesulfonamide hydrochloride.	32,000	16,000	64,000	400	64,000	400	64,000	> 8,000
<i>p</i> -( $\alpha$ -aminoethyl)-benzenesulfonamide hydrochloride....	8,000	2,000	32,000	200	32,000	100	64,000	100
<i>p</i> -( $\beta$ -aminoethyl)-benzenesulfonamide hydrochloride....	<100	<100	<100	<100	<100	<100	<100	<100
<i>p</i> -Aminomethylbenzenesulfonamide sulfosalicylate... ..	16,000	16,000	32,000	400	64,000	2,000	4,000	400
<i>p</i> -Aminomethylbenzenesulfonamide mandelate. . . . .	8,000	8,000	64,000	32,000	64,000	2,000	64,000	> 2,000
<i>p</i> -Aminomethylbenzenesulfonamide methane-bis(2-hydroxy-3-naphthoate) .....	16,000	8,000	64,000	> 800	64,000	> 1,000	16,000	1,000
Sodium sulfathiazole.....	<100	<100	<100	<100	<100	<100	<100	<100
Sodium sulfadiazine .....	<100	<100	<100	<100	<100	<100	<100	<100

Bs = Bacteriostatic; Bc = Bactericidal.

Figures preceded by < indicate highest drug concentration tested (1:100) proved ineffective.

Figures followed by > indicate lowest drug concentration tested (1:64,000) proved effective.

TABLE 2

*Highest dilution of compound showing antibacterial effect against several strains of anaerobic bacteria*

COMPOUND	C. NOVYI		C. TETANI		C. CHAUVEI		C. BOTULINUM	
	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc
<i>p</i> -Aminomethyl-benzenesulfonamide hydrochloride. .	64,000	> 32,000	64,000	> 8,000	32,000	4,000	64,000	> 8,000
<i>p</i> -( $\alpha$ -aminoethyl)-benzenesulfonamide hydrochloride. . .	32,000	400	32,000	400	800	200	800	100
<i>p</i> -( $\beta$ -aminoethyl)-benzenesulfonamide hydrochloride . .	<100	<100	<100	<100	<100	<100	<100	<100
<i>p</i> -Aminomethylbenzenesulfonamide sulfosalicylate . . . .	16,000	2,000	64,000	8,000	4,000	400	4,000	100
<i>p</i> -Aminomethylbenzenesulfonamide mandelate.....	64,000	> 32,000	32,000	8,000	32,000	2,000	64,000	> 2,000
<i>p</i> -Aminomethylbenzenesulfonamide methane-bis(2-hydroxy-3-naphthoate).....	32,000	8,000	64,000	400	8,000	2,000	16,000	200
Sodium sulfathiazole.....	<100	<100	<100	<100	<100	<100	<100	<100
Sodium sulfadiazine.....	<100	<100	<100	<100	<100	<100	<100	<100

See below table 1.

In studies using several strains of anaerobes obtained from Major Champ Lyons, which were recently isolated from war-wound infections, *p*-aminomethylbenzenesulfonamide hydrochloride proved to be bactericidal to *Clostridium welchii* in dilutions of 1:2,000 to 1:4,000 and to *C. tetani* in dilutions of 1:8,000 to 1:16,000. Under the same experimental conditions sodium sulfathiazole and sodium sulfadiazine gave but a suggestion of an antibacterial effect against the organisms in the highest concentration used (1:100).

II. *Effects of compounds against gram-positive cocci.* The antibacterial effects of the *p*-aminomethylbenzenesulfonamide derivatives were compared with sulfathiazole and sulfadiazine against beta hemolytic streptococcus C-203, types I, II, and III pneumococci, a viridans streptococcus, and *Streptococcus faecalis*. Serial dilutions of the sulfonamides were made in veal infusion broth containing 0.15 per cent glucose and bacto-peptone, and adjusted to pH 7.4. The drug-broth solutions were distributed in 10-ml amounts in cotton-plugged test tubes and sterilized at 10 pounds for 10 minutes. Upon cooling, to each tube was added aseptically 0.1 ml of sterile, normal horse serum and 0.2 ml of a 1:1,000 dilution of a 24-hour culture of test organism. Following the schedule described before, observations were made of the incubated tubes with the necessary subcultures to determine the possible bactericidal effects of the compounds.

The results of this study are presented in tables 3 and 4. From the data given in table 3, it is evident that all but one of the *p*-aminomethylbenzenesulfonamide compounds tested showed an antibacterial effect against  $\beta$ -hemolytic streptococcus and the pneumococci. *Para*-( $\beta$ -aminoethyl)-benzenesulfonamide again proved to be ineffective as an antibacterial agent in the highest drug concentration tested, confirming in part the results obtained when testing this compound against anaerobic bacteria (tables 1 and 2). On the basis of molar equivalents the salts of *p*-aminomethylbenzenesulfonamide gave comparable degrees of bacteriostatic and bactericidal effects against the test organisms and further approximated the activity of sodium sulfathiazole and sodium sulfadiazine. *Para*-( $\alpha$ -aminoethyl)-benzenesulfonamide was somewhat less active than the salts, indicating that the presence of the ethyl group in the compound materially reduced the activity of the drug.

The *p*-aminomethylbenzenesulfonamide derivatives proved to be definitely inhibitory, and in some instances bactericidal, to the strains of viridans streptococci studied (table 4). The *p*-( $\beta$ -aminoethyl) analogue of *p*-aminomethylbenzenesulfonamide, sodium sulfathiazole, and sodium sulfadiazine, in the highest concentrations tested, were entirely ineffective against the organisms mentioned. *Para*-aminomethylbenzenesulfonamide hydrochloride, its mandelate, and methane-bis(2-hydroxy-3-naphthoate) salts were the only sulfonamides studied which gave any suggestion of an antibacterial action against the two strains of *Streptococcus faecalis*.

III. *Effects of compounds against Staphylococcus aureus and Pseudomonas aeruginosa.* The antibacterial effects of *p*-aminomethylbenzenesulfonamide hydrochloride were compared with sodium sulfathiazole and sodium sulfadiazine against several strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

TABLE 3

*Highest dilution of compound showing antibacterial effect against beta hemolytic streptococcus and pneumococci*

COMPOUND	$\beta$ -HEMOLYTIC STREPTOCOCCUS		PNEUMOCOCCUS TYPES					
	Bs	Bc	I		II		III	
			Bs	Bc	Bs	Bc	Bs	Bc
<i>p</i> -Aminomethyl-benzene-sulfonamide hydrochloride.....	32,000	8,000	32,000	4,000	32,000	16,000	32,000	8,000
<i>p</i> -( $\alpha$ -aminoethyl)-benzene-sulfonamide hydrochloride.....	2,000	1,000	4,000	<1,000	4,000	2,000	<1,000	<1,000
<i>p</i> -( $\beta$ -aminoethyl)-benzene-sulfonamide hydrochloride.....	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000
<i>p</i> -Aminomethylbenzenesulfonamide sulfosalicylate..	16,000	4,000	32,000	4,000	16,000	4,000	16,000	2,000
<i>p</i> -Aminomethylbenzenesulfonamide mandelate...	16,000	4,000	32,000	4,000	16,000	8,000	16,000	4,000
<i>p</i> -Aminomethylbenzenesulfonamide methane-bis(2-hydroxy-3-naphthoate)..	16,000	8,000	32,000	8,000	32,000	8,000	32,000	4,000
Sodium sulfathiazole.....	8,000	4,000	32,000	4,000	32,000	8,000	32,000	4,000
Sodium sulfadiazine....	2,000	<1,000	8,000	1,000	4,000	<1,000	16,000	<1,000

See below table 1.

TABLE 4

*Highest dilution of compound showing antibacterial effect against viridans streptococci and Streptococcus faecalis*

COMPOUND	VIRIDANS STREPTOCOCCI				S. FAECALIS			
	74		1454		7080		4080	
	Bs	Bc	Bs	Bc	Bs	Bc	Bs	Bc
<i>p</i> -Aminomethyl-benzenesulfonamide hydrochloride . . . . .	16,000	400	8,000	4,000	400	<100	400	<100
<i>p</i> -( $\alpha$ -aminoethyl)-benzenesulfonamide hydrochloride. . . . .	800	<100	1,000	<100	<100	<100	<100	<100
<i>p</i> -( $\beta$ -aminoethyl)-benzenesulfonamide hydrochloride . . . . .	<100	<100	<100	<100	<100	<100	<100	<100
<i>p</i> -Aminomethylbenzenesulfonamide sulfosalicylate..	8,000	100	8,000	1,000	<100	<100	<100	<100
<i>p</i> -Aminomethylbenzenesulfonamide mandelate.....	8,000	100	8,000	2,000	200	<100	200	<100
<i>p</i> -Aminomethylbenzenesulfonamide methane-bis(2-hydroxy-3-naphthoate).....	8,000	800	8,000	1,000	800	100	800	200
Sodium sulfathiazole.....	<100	<100	<100	<100	<100	<100	<100	<100
Sodium sulfadiazine.....	<100	<100	<100	<100	<100	<100	<100	<100

See below table 1.

in the presence of Brewer's fluid thioglycollate medium. A 4-mm loopful of undiluted fluid thioglycollate medium cultures was used as the inoculum in all instances.

The results of this study showed that a 1 per cent concentration of *p*-aminomethylbenzenesulfonamide hydrochloride is bactericidal for *Staphylococcus aureus*, while the same concentration of sodium sulfathiazole is bacteriostatic. Sodium sulfadiazine in the same test was entirely ineffective against the staphylococci. The three compounds in concentrations of 1:200 to 1:400 were all equally effective as bacteriostatic agents against *Pseudomonas aeruginosa*.

IV. *Effects of p-aminobenzoic acid and p-aminomethylbenzoic acid upon p-aminomethylbenzenesulfonamide activity.* Mention was made earlier that *p*-aminobenzoic acid failed to inhibit the action of *p*-aminomethylbenzenesulfonamide *in vitro*. Schreus (1942) observed this negative effect in plate cultures of *C. welchii* containing the acid and *p*-aminomethylbenzenesulfonamide, and also noted that the sulfonamide was active against the organism in liver peptone medium, although the latter inhibits most other sulfonamides. The inability of *p*-aminobenzoic acid to act as an antagonist to *p*-aminomethylbenzenesulfonamide action has been confirmed by McKee *et al.* (1944), Clark (1944), and Domagk (1944).

In attempting to explain the possible mechanism of action of *p*-aminomethylbenzenesulfonamide against anaerobic bacteria, Schreus, moreover, postulated that while *p*-aminobenzoic acid is not concerned with the action of the sulfonamide, the analogue of *p*-aminobenzoic acid, namely, *p*-aminomethylbenzoic acid, is the metabolite which is in competition with *p*-aminomethylbenzenesulfonamide for the growth of the organisms. This would be a logical deduction from the Woods-Fildes theory (1940) on the mechanism of the interfering action of *p*-aminobenzoic acid upon the antibacterial effects of the commonly known sulfonamides (sulfanilamide, sulfapyridine, sulfathiazole, etc.) against aerobic bacteria.

The possible antagonistic actions of *p*-aminomethylbenzoic acid, as well as *p*-aminobenzoic acid, upon the antibacterial effects of the *p*-aminomethylbenzenesulfonamide derivatives against *C. welchii* were studied by the following *in vitro* method. Serial dilutions of the compounds were made in Brewer's thioglycollate medium containing a 1:5,000 concentration of one of the benzoic acids. This amount of *p*-aminobenzoic acid has been found to inactivate completely the antibacterial effects of all the commonly known sulfonamides (sulfanilamide, etc.). Suitable controls containing the *p*-aminomethylbenzenesulfonamide compounds without the benzoic acids, as well as the latter alone, in nutrient medium were maintained throughout the tests. Sterilization of the test solutions, amount of inoculum, and observation of the incubated tubes followed in detail the schedule given in the first section of this report. The results of a typical series of these tests are presented in table 5.

It is evident from the data given in the table that neither *p*-aminobenzoic acid nor *p*-aminomethylbenzoic acid proved to be antagonistic to the antibacterial effects of *p*-aminomethylbenzenesulfonamide compounds against the anaerobe,



*C. welchii*. Although not presented here, data are available which indicate that under the same experimental conditions results similar to those presented in table 5 were obtained against the other anaerobic bacteria used in these investigations.

The effects of *p*-aminobenzoic acid and *p*-aminomethylbenzoic acid upon the antibacterial actions of *p*-aminomethylbenzenesulfonamide hydrochloride against  $\beta$ -hemolytic streptococcus C-203 in an aerobic medium seemed to merit consideration. Furthermore, since an antagonistic action of *p*-aminomethylbenzoic acid to *p*-aminomethylbenzenesulfonamide was not demonstrated (table 5), it appeared worthwhile to compare the *in vitro* effects of the latter benzoic acid with *p*-aminobenzoic acid upon one of the commonly known sulfonamides, namely, sulfathiazole. Concentrations of 1:5,000 of the benzoic acids were added to veal glucose broth. These solutions, as well as broth lacking the acids, were used to prepare serial dilutions of *p*-aminomethylbenzenesulfonamide

TABLE 5

Effects of *p*-aminobenzoic acid and *p*-aminomethylbenzoic acid upon antibacterial actions of *p*-aminomethylbenzenesulfonamide derivatives against *Clostridium welchii*

SULFONAMIDE DILUTION	<i>p</i> -AMINOMETHYLBENZENESULFONAMIDE*												<i>p</i> -( $\alpha$ -AMINOETHYL)-BENZENESULFONAMIDE											
	Control				PAB				PAMB				Control				PAB				PAMB			
	24	48	72	S	24	48	72	S	24	48	72	S	24	48	72	S	24	48	72	S	24	48	72	S
1:1,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1:2,000	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2	+	1	3	4	—	1	1	4	—
1:4,000	0	0	0	0	0	0	0	0	0	0	0	0	2	4	4	—	2	4	4	—	2	4	4	—
1:8,000	0	0	0	+	0	0	0	0	0	0	0	0	3	4	4	—	3	4	4	—	4	4	4	—
1:16,000	0	1	1	+	1	1	1	+	1	1	1	+	4	4	4	—	4	4	4	—	4	4	4	—
1:32,000	1	4	4	—	1	2	4	—	1	4	4	—	4	4	4	—	4	4	4	—	4	4	4	—
1:64,000	2	4	4	—	3	4	4	—	3	4	4	—	4	4	4	—	4	4	4	—	4	4	4	—

\* = The sulfonamides were used as the hydrochlorides. PAB = *p*-aminobenzoic acid 1:5,000; PAMB = *p*-aminomethylbenzoic acid 1:5,000; 0 = no visible growth; 1 to 4 = slight to luxuriant growth; + = growth in subculture tube; — = no test made.

hydrochloride and sodium sulfathiazole. Distribution of the test solutions and inoculation and incubation of the media followed in detail the foregoing procedure. The results of this study are presented in table 6. The data given indicate that *p*-aminobenzoic and *p*-aminomethylbenzoic acids are not antagonistic to the antibacterial effects of *p*-aminomethylbenzenesulfonamide hydrochloride against  $\beta$ -hemolytic streptococcus in an aerobic medium. Furthermore, while *p*-aminobenzoic acid proved to neutralize completely the antibacterial effects of sulfathiazole against the same test organism, this antagonistic action against the latter sulfonamide was entirely lacking when *p*-aminomethylbenzoic acid was used.

In the two previous tests a uniform concentration (1:5,000) of the benzoic acids was used in the presence of increasing dilutions of the sulfonamides. Since it appeared possible that higher concentrations of the acids might show evidence of a neutralizing action against *p*-aminomethylbenzenesulfonamide activity, the

following study was carried out. Procaine hydrochloride was included in this test as another sulfonamide (sulfanilamide, etc.) antagonist. The benzoic acids and local anesthetic were tested in molar concentrations of  $M/1,000$ ,  $M/2,000$ ,  $M/4,000$ , and  $M/8,000$  against serial dilutions of *p*-aminomethylbenzenesulfonamide hydrochloride.  $\beta$ -hemolytic streptococcus C-203 was used as the test organism in veal glucose broth medium containing serum as before.

Procaine hydrochloride and *p*-aminomethylbenzoic acid in all the concentrations given were found to be entirely inactive as antagonists against the antibacterial effects of *p*-aminomethylbenzenesulfonamide hydrochloride upon the test organism. There was a suggestion, however, of an apparent interfering action of the two highest concentrations of *p*-aminobenzoic acid against the sulfonamide. In view of the complete absence of an antagonistic action by procaine hydrochloride against *p*-aminomethylbenzenesulfonamide in the same test, it might be assumed that the results obtained with *p*-aminobenzoic acid were due to a

TABLE 6

*Effects of p-aminobenzoic acid and p-aminomethylbenzoic acid upon antibacterial actions of p-aminomethylbenzenesulfonamide and sulfathiazole against beta hemolytic streptococcus*

SULFONAMIDE DILUTION	<i>p</i> -AMINOMETHYLBENZENESULFONAMIDE												Na SULFATHIAZOLE											
	Control				PAB*				PAMB				Control				PAB				PAMB			
	24	48	72	S	24	48	72	S	24	48	72	S	24	48	72	S	24	48	72	S	24	48	72	S
1:1,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	—	0	0	0	0
1:2,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	—	0	0	0	0
1:4,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	—	0	0	0	+
1:8,000	0	0	0	0	0	0	0	+	0	0	0	0	1	4	4	—	4	4	4	—	0	1	4	—
1:16,000	0	0	0	+	0	1	4	—	0	0	0	+	1	4	4	—	4	4	4	—	0	2	4	—
1:32,000	2	4	4	—	2	4	4	—	2	4	4	—	2	4	4	—	4	4	4	—	2	4	4	—

\* See below table 5.

growth-stimulating factor of the acid upon the organisms and not associated with the mechanism of action of *p*-aminobenzoic acid against sulfanilamide and related compounds.

Evidence of the inability of *p*-aminomethylbenzoic acid to interfere with the antibacterial effects of *p*-aminomethylbenzenesulfonamide compounds against anaerobic and aerobic organisms *in vitro* invalidates the theory advanced by Schreus that the acid is an essential metabolite for bacteria.

#### SUMMARY

The sulfosalicylate, mandelate, and methane-bis(2-hydroxy-3-naphthoate) salts of *p*-aminomethylbenzenesulfonamide compare in activity with the hydrochloride in inhibiting and destroying anaerobic organisms. Whereas the *p*-( $\alpha$ -aminoethyl) analogue of *p*-aminomethylbenzenesulfonamide shows some evidence of an antibacterial effect upon anaerobes, this same action is entirely lacking when *p*-( $\beta$ -aminoethyl)-benzenesulfonamide is used.

Molar equivalents of the salts of *p*-aminomethylbenzenesulfonamide are equally effective against  $\beta$ -hemolytic streptococcus C-203 and pneumococci. These compounds approximate the activity of sodium sulfathiazole and sodium sulfadiazine against the organisms mentioned.

*Para*-aminomethylbenzenesulfonamide salts are definitely inhibitory to viridans streptococci *in vitro*. The compounds, furthermore, give a suggestion of an antibacterial action against *Streptococcus faecalis*. Under similar experimental conditions sodium sulfathiazole and sodium sulfadiazine are entirely ineffective against these bacteria.

*Para*-aminomethylbenzenesulfonamide hydrochloride is bactericidal for *Staphylococcus aureus*, while the same concentration of sodium sulfathiazole is bacteriostatic and sodium sulfadiazine entirely ineffective. The three compounds were equally effective against *Pseudomonas aeruginosa*.

Neither *p*-aminobenzoic acid nor *p*-aminomethylbenzoic acid, an analogue of the former compound which also contains the same *p*-aminomethyl group present in *p*-aminomethylbenzenesulfonamide, interferes with the antibacterial action of *p*-aminomethylbenzenesulfonamide derivatives.

These findings do not support the theory advanced by Schreus that *p*-aminomethylbenzoic acid is an essential metabolite for bacterial growth.

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# CORYNEBACTERIUM EQUI IN CHRONIC PNEUMONIA OF THE CALF

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It has been common practice in the classification of bacteria to identify pathogenic species with the type of disease caused, or with the plant or animal from which the bacteria have been first isolated. The use of the latter scheme, of nomenclature is now frequently confusing, because many pathogens originally considered to be confined in their disease-producing activities to a single plant or animal species have since been found to produce natural infections in various unrelated species. An example is the diphtheroid bacillus isolated by Magnusson (1923) in Sweden and named by him *Corynebacterium equi* because it was recovered from purulent pneumonia in foals. This organism was likewise isolated from purulent pneumonia among foals in Germany by Miessner and Wetzel (1923) and Lütje (1923). In 1931 it was recovered from pneumonic foals in the United States by Dimock and Edwards. These findings lent weight to the use of a specific name derived from the animal for which the organism seemed to be naturally infectious. However, the name *C. equi* now appears to be a misnomer in view of the fact that this diphtheroid bacterium has also been isolated from cervical lymph glands and pulmonary lesions of swine by Bendixen and Jepsen (1939) and from the submaxillary lymph nodes of swine by Karlson, Moses, and Feldman (1940).

Some time ago the respiratory organs of a 6-month-old calf, dead of chronic pneumonia, were submitted to this laboratory for examination. Gross observation of cross sections of the lungs revealed marked hepatization and many small mucopurulent lesions approximately 3 mm in diameter, which were uniformly distributed throughout the lung tissue. The bronchi and trachea were likewise filled with pus, principally in the form of a mucopurulent exudate.

Microscopic examination of stained smears of the pus revealed an enormous number of short rod or coccoid forms. Many of these appeared in chains and presented the appearance of streptococci. The cells stained gram-positively and measured from 1.0 to 1.5 microns in length by 0.8 to 1.0 microns in width.

Microscopic findings tended to create the impression that infection had probably been induced by streptococci which had undergone dissociation. However, this concept was discarded following culture of the pus. Blood agar plates streaked with material from the lesions yielded, after 24 hours at 37 C, small, moist, slightly raised, nonhemolytic colonies with smooth edges. These colonies increased in size with continued incubation, became finely granular, and presented an orange-pink color. The colonial growth on the plates gave evidence that the bacteria had been present in the pus-filled lesions in pure culture. The

organism grew readily at 37 C on Difco proteose no. 3 and beef extract agars, as well as on blood agar.

Nutrient broth inoculated with pure cultures obtained from plates produced organisms of a highly pleomorphic nature. They no longer exhibited the short

TABLE 1

*Description of Corynebacterium equi Magnusson\**

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*Corynebacterium equi* Magnusson (Arch. wiss. prakt. Tierheilk., 50, 22, 1923-24).

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Rods: 1.0 to 1.5 by 0.8 to 1.2 microns; occurring in exudates as coccoid forms, singly, in pairs and chains; on solid media and in liquids as coccoid or elongated, often filamentous rods, club-shaped, striated or granular; gram-positive; nonmotile; nonsporeforming.

Gelatin stab: No liquefaction.

Agar colonies: Moist, slightly raised, finely granular, entire; growth occurs within 24 hours; increase in size with continued incubation; develop light tan, salmon to orange-pink or red pigment.

Agar slant: Fairly abundant, somewhat beaded growth.

Blood agar: No hemolysis.

Loeffler's serum medium: Moderate growth; no liquefaction.

Broth: Fairly abundant growth with uniform turbidity; pellicle absent.

Litmus milk: No change.

Potato slant: Scant growth; yellowish-tan pigment becoming orange after one or more weeks.

Indole is not formed.

Nitrates reduced to nitrites.

No acid from carbohydrates.

Aerobic.

37 C.

Source: Spontaneous pneumonia in foals, lymph node infections and pneumonia in swine, chronic pneumonia in calves.

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\* Descriptive information based on observations made during present investigation as well as on the previously published reports of others.

rod, coccoidal, or streptococcal forms observed in the pus. Instead, the majority of the cells were club-shaped, elongated, or filamentous, and striated or granular. They were gram-positive, nonacid-fast and nonmotile. On agar, the cells were less elongated and some coccoidal forms were observed.

The organism was found to grow readily in various differential media. Nutrient broth became uniformly cloudy and nitrates were reduced. No biochemical changes were observed in litmus milk, tryptone, carbohydrate media, lead acetate agar, Loeffler's medium, or gelatin, although growth occurred in all (table 1). The organism was as susceptible to heat as are most nonsporulating bacteria, being destroyed at 58 C in 20 minutes. It was killed by 1 per cent phenol in 10 minutes, but not by full-strength chlorox in the same period of time.

Attempts to establish the organism in mice, guinea pigs, and rabbits by intratracheal inoculation failed, even though these animals received from 0.10 to 0.25 ml of a cloudy broth culture. One rabbit injected intradermally with 0.1 ml of broth culture developed within 5 days an abscess at the site of inoculation, approximately 1 cm in diameter. Stained smears of the pus again presented the coccoidal and streptococcal forms observed originally in the lesions of the organs of the calf. On culture, however, the diphtheroidal forms were again established.

The organism under discussion was identified as *Corynebacterium equi*. This conclusion was reached because it performed typically, both morphologically and in culture, according to the description of *C. equi* as set forth by Karlson, Moses, and Feldman (1940) and by Kelser and Schoening (1943).

In view of the fact that *C. equi* has been proved pathogenic to swine as well as to the horse, and has now been isolated from an infection in a calf, it is possible that this organism is more frequently involved in livestock diseases than has been generally recognized. Hence, the limited recognition given the species in *Bergey's Manual of Determinative Bacteriology* (1939) no longer suffices. With the inclusion in bacterial classification of a more lengthy description of this organism, it might be well to consider changing the species name to one more appropriate than that employed at present. The writer is fully aware of the International Rules of Botanical Nomenclature covering changes or modifications of names and is opposed to frequent changes in nomenclature because of the confusion created in the literature. However, it seems more logical to employ a specific name for a pathogenic bacterium which describes or suggests the nature of the infection induced than to use the name of the discoverer or the host from which the species was first isolated. In view of the apparent unsuitability of the name, *C. equi*, it might be well to consider one such as *Corynebacterium purulentus*. This would suggest the nature of the infection induced by the species without regard to the animal host.

#### SUMMARY

An organism, identified as *Corynebacterium equi*, was isolated from purulent lesions in the lungs of a calf dead of chronic pneumonia of several months' duration. No other organism was associated with it in the lesions. The organism was pathogenic to the rabbit upon intradermal but not upon intratracheal inoculation.

The suggestion is made that the present name, *C. equi*, be changed to one descriptive of the pathology of the disease, since the organism is infectious for other animals besides the horse.

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# STARCH DIGESTION BY *VIBRIO CHOLERA*E IN STRONGLY AERATED MEDIA<sup>1</sup>

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The ability of *Vibrio cholerae* to digest starch was recognized very early in the study of this microorganism. Bitter (1886), Fermi (1890), and Wherry (1905) were among the first to discuss this amylolytic activity, and their observations have been passed on more or less unchanged. It is generally stated that *V. cholerae* rapidly digests starch with the formation of acid but not of gas. Although this statement is true enough when applied to the usual fermentation tube, the work reported here indicates that an adequate amount of aeration modifies this phenomenon considerably.

The starch metabolism of the vibrio is not without some practical interest as it has frequently been proposed as a differential characteristic for rapid identification of carriers, tainted water supplies, etc. Gordon (1906) suggested the use of a medium in which the acidification of an alkaline starch medium would be indicated by the color change of litmus. Gibson (1916) and, independently, Lange (1916) made use of solid media in which the turbidity imparted by the starch would give way to a zone of clearing around vibrio colonies. Kodama and Takeda (1922) proposed a liquid medium in which disappearance of undissolved starch and failure to obtain a color reaction with iodine were to be diagnostic criteria for the presence of the cholera vibrio in suspected inocula. The work of Gildemeister and Herzberg (1923) and of Nobechei (1925), however, demonstrated that amylolysis is insufficiently specific to be used as a differentiating characteristic.

It is of interest, in connection with the present communication, that Lange (1916) stated that he was then engaged in research directed toward enrichment of cultures by means of aeration, which might be reported on later. Apparently, this was never done.

## EXPERIMENTAL

Wherry (1905) believed that the acid derived from starch did not inhibit the growth of *V. cholerae*, as did the acid which accumulated in glucose or maltose media. Since the method developed at the Biochemical Research Foundation for the production of "Direct Vaccine" (Jennings and Linton, 1944b) is premised on the evasion of the deleterious effects of such acid, we investigated this claim.

The "Salts-C-D" medium originally used (Linton and Jennings, 1944) was subject to the limiting effect of the acid. Accordingly, we used this medium,

<sup>1</sup> The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Biochemical Research Foundation of the Franklin Institute.



substituting 1 per cent starch for the 0.3 per cent glucose previously employed. The medium thus became 0.5 per cent  $(\text{NH}_4)_2\text{SO}_4$ , 0.075 per cent  $\text{K}_2\text{HPO}_4$ , and 0.01 per cent  $\text{MgSO}_4$  to which 15 ml of CD (tryptic digest of a 10 per cent suspension of casein) and 10 grams of soluble starch per liter were added. The pH was adjusted to 8.0 with 25 per cent NaOH, and the medium was inoculated with a young culture of *V. cholerae* (strain 35). Vigorous aeration provided by a cloth bubbler of the type used in production of the BRF direct vaccine (Jennings and Linton, 1944c) was continued throughout the growth period.

*Starch digestion.* Samples removed at intervals during growth were tested for starch by the addition of an equal volume of Lugol's solution. Failure to obtain any further starch-iodine color reactions after about 24 hours verified the depolymerization of the starch.

Growth was rapid and heavy, though the physical nature of the medium interfered with accurate estimation of the population. A thick persistent foam held great numbers of the organisms (Rahn and Richardson, 1942). As the starch digestion proceeded the foam subsided, but the organisms remained coherent and floated about in the medium as heavy gelatinous masses. On purely subjective grounds the growth was estimated to be equal to that obtained in similar circumstances with glucose. In passing it might be well to mention that the lack of dispersion renders the culture totally unsuitable for vaccine.

*pH changes.* Contrary to expectation the pH, as recorded by means of glass electrodes incorporated in the culture vessel, did not decrease at any time during growth. Jennings and Linton (1944a) have shown that if glucose equivalent to that which should be released during the saccharification of the starch had been originally incorporated in the medium, sufficient acid would have accumulated to depress the pH to 5.5, thus stopping further development. In all aerated starch cultures, however, the pH was found to remain constant or to rise slightly.

*Saccharification of the starch.* Repeating the original experiment, samples withdrawn at intervals were also tested for the presence of reducing sugars. At no time was there a sufficient accumulation of glucose to bring about a positive reaction with Benedict's qualitative reagent. In view of the disappearance of the starch and the failure of the culture to become acid this seemed somewhat surprising. Although it did not seem probable that sufficient energy for the production of the heavy crop of vibrios could be derived from degradation of the starch to dextrin, a test was made. No dextrin color reaction was obtained when iodine was added to the completed culture, and it was only by precipitating the alcohol-insoluble material from a much concentrated filtrate of the culture that even a small amount of dextrin could be demonstrated. This precipitate, taken up in a minimum amount of water, did indeed give a faint mahogany color with iodine.

The glucose, if formed, must have been digested with considerable rapidity, and the usual acid residues must have been attacked as well.

*Digestion of acid residue.* In order to confirm the plausibility of this explanation, salts-C-D media in which the glucose was replaced by (a) 1 per cent sodium formate, (b) 1 per cent sodium acetate, and (c) 1 per cent sodium lactate were

prepared. These substrates were inoculated and aerated as usual. No growth was obtained in the formate medium, but the other two gave rise to satisfactory crops of vibrios. The acetate medium developed a culture having a turbidity of about 2,000 ppm of silicate, whereas the lactate culture was about twice as heavy, comparing favorably with the growth which is obtained when glucose is used.

When these two media were inoculated and incubated *without aeration*, growth occurred as a very delicate surface film, indicating that thorough saturation with air is a prerequisite for digestion of the acid by-products of glucose metabolism.

*Disadvantages of the lactate substrate.* Although the use of sodium lactate in place of glucose removed the objectionable feature of acid formation, the accumulation of sodium ion proved equally bad. Various methods of circumventing this difficulty were tried, without success. Adding both glucose and sodium lactate to the medium did, as anticipated, result in an initial depression of the pH but the terminal alkalinity still approached 10 without any great increase in crop. Ammonium lactate, either incorporated as the syrupy compound, or formed by bubbling air passed over heated  $(\text{NH}_4)_2\text{CO}_3$  through the medium to which lactic acid had been added, would not support growth of the vibrio unless the pH was maintained in the neighborhood of neutrality. This could be accomplished by the use of the bicarbonate- $\text{CO}_2$  balance system employed in BRF cultures (Jennings and Linton, 1944b), but the product was in no way superior to that obtained with glucose, and the growth was not so good. It is probable that under these conditions the vibrio is constantly supplied with sodium lactate, so that the ability of the organism to utilize the ammonium salt remains unproved.

#### DISCUSSION

Although it is true of the usual stagnant culture that the digestion of starch by *V. cholerae* is accompanied by accumulation of acid in the medium, the presence of sufficient oxygen may modify this course of events. Perhaps certain discrepancies between the observations of different workers may be resolved by this fact. For example, if Gildemeister and Herzberg (1923) were using a deeper medium than Kodama and Takeda (1922), the failure of the former authors to observe bleaching of the dextrin-iodine complex as reported by the latter might be attributed to a difference in pH. The shallow culture, receiving more air, would tend to be acidified more slowly, or not at all, and the final culture might then be sufficiently alkaline to interfere with the color reaction. It may also be that Wherry's (1905) belief in the relative innocuousness of the acid derived from starch may have been founded on similar grounds.

In any event, the concentration of air in the medium must be taken into account in studying the acid production from carbohydrates, especially in cultural identification procedures.

#### SUMMARY

Heavy crops of *Vibrio cholerae* may be obtained in liquid media containing starch as the source of energy for reproduction, without accumulation of acid by-products, *provided* that the culture is efficiently aerated. Good growth may

also be obtained when sodium lactate is used as the energy food. Neither of these substrates is so satisfactory as the previously described BRF medium for vaccine production, however.

The failure to acidify starch media is at variance with the accepted report of the action of *V. cholerae* in such a substrate. When adequate aeration is provided it appears that the digestion of simple sugars outstrips the production of such nutrients by the saccharification of the dextrins derived from starch. Under these conditions the acid radicals derived from the sugar are in turn attacked and do not accumulate to acidify the culture. Digestion of the acetate and lactate radicals appears to be a highly aerobic process which does not take place to any great extent so long as glucose is available.

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# SOME EFFECTS OF METALLIC IONS ON THE METABOLISM OF *AEROBACTER AEROGENES*<sup>1</sup>

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The effects of traces of metallic ions on bacterial metabolism have not been very extensively investigated. The obstacle to research in this field is the lack of bacteriologically complete media which are relatively free of metallic ions. Adsorption methods for freeing the media from most of the metallic ions have been successful in producing media for studies on the effects of metallic ions on the metabolism of molds and yeasts. These methods have usually proved to be unsatisfactory for most bacteriological studies, as bacteria are more sensitive to small quantities of metallic ions in the medium than are either the molds or the yeasts. Perhaps the simplest method to overcome this obstacle is to use extremely pure ingredients in the preparation of the medium. The purification of these ingredients is very laborious, and the resulting medium is often unsatisfactory.

A method that can be used for this purpose is to pass the nonionic portion of the medium over a cationic exchange material. This method has been successfully used in the preparation of media low in metallic ions for studies on the effects of added metallic ions on the citric acid fermentation (Perlman *et al.*, 1944). The following experiments will show that it can be used to purify media for similar studies on bacterial metabolism.

Best results can be obtained with such a method on media containing only carbohydrate and purified salts. Among the organisms that will grow well on such media are members of the colon-aerogenes group. Waring and Werkman (1943a, 1943b) have included organisms of this group in their studies on the growth of bacteria on iron-deficient media. Recently they have reported on investigations concerning the effects of iron deficiency on the enzyme systems of *Aerobacter indologenes* (1944).

A similar organism, *Aerobacter aerogenes*, was used in the following experiments. This organism grows well anaerobically on media consisting of carbohydrate and salts. Thompson (1942) and Burkholder and McVeigh (1942) have shown that this organism is capable of synthesizing appreciable quantities of B vitamins during its growth. Hence, when considering the effects of metallic ions on the metabolism of this organism, synthesis of B vitamins as well as apparent changes in fermentation mechanism should be considered.

## METHODS

A strain of *Aerobacter aerogenes*, obtained from the Northern Regional Research Laboratory and designated in that collection as *A. aerogenes* 199, was used

<sup>1</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

in these experiments. Stock cultures of this organism were carried on glucose yeast-extract agar. Inocula for the fermentations were prepared by transferring the organism from the stock culture tubes to test tubes containing 10 ml of medium of the same composition as the medium to be fermented. At least two successive transfers were made on the liquid medium with 18-hour incubation intervals before use as inoculum for the fermentations. The inoculum volume varied between 0.5 and 1 per cent of the fermentation volume. Both the fer-

TABLE 1  
*Composition of media*

	MEDIUM A	MEDIUM B	MEDIUM C
	g/L	g/L	g/L
Glucose.....	20	30	20
Peptone.....	10	0	0
KH <sub>2</sub> PO <sub>4</sub> .....	0.5	0.5	0.5
Na <sub>2</sub> HPO <sub>4</sub> .....	2.0	2.0	2.0
MgCl <sub>2</sub> .....	0.1	0.1	0.1
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.05	0.05	0.05
Urea.....	3.0	3.0	3.0
pH after autoclaving .....	6.25	6.10	6.05
	μg/L	μg/L	μg/L
Trace metal analysis			
Aluminum.....	*	27.3	8.1
Calcium.....	5,800	88.5	14.6
Cadmium.....	*	3.2	1.4
Cobalt.....	*	5.5	2.1
Copper.....	*	1.8	1.6
Iron.....	299	5.4	less than 1.0
Lead.....	*	58.3	3.8
Manganese.....	750	30.8	6.4
Molybdenum.....	19	6.3	2.2
Zinc.....	*	31.3	less than 1.0

\* Not determined; would be more than medium B.

mentations and the inoculum were incubated at 30 C. All fermentations were 48 hours in duration.

The composition of the media used in the experiments is presented in table 1. In the preparation of medium C, the cp glucose was dissolved in distilled water and the solution passed over a bed of "zeo-karb H," a cationic exchange material obtained from the Permutit Corporation, New York. This bed had a volume of 200 ml, and the material was operated on a hydrogen cycle (sulfuric acid). The solution was passed over the bed at a rate of 10 ml per minute. The reaction of the solution changed from pH 6.9, before the treatment, to pH 4.8 after the treatment. The urea and inorganic salts indicated in table 1, which had been recrystallized at least twice, were added to the treated solution. The sources of

the metallic ions added to the fermentations mentioned in table 3 were:  $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$  (Fe);  $\text{Al}_2(\text{SO}_4)_3 \cdot 36\text{H}_2\text{O}$  (Al);  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (Cu);  $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$  (Mn);  $\text{K}_2\text{Cr}_2\text{O}_7$  (Cr);  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (Zn). These salts were all of reagent grade.

The glassware used in the preparation of the media and containers for the fermentations were cleaned with dichromate cleaning solution. They were rinsed with distilled water at least five times to remove any traces of the cleaning solution. Two hundred ml of medium were placed in pyrex 500-ml Erlenmeyer flasks which were covered with 100-ml pyrex beakers. The medium was autoclaved for 30 minutes at 120 C.

One liter of medium A and 6 liters each of medium B and medium C were ashed at 500 C. Two ml of concentrated sulfuric acid were added during the concentration of these solutions to prevent volatilization of metallic elements present during the ashing. The ash was dissolved in a mixture of dilute HCl and  $\text{HClO}_4$  and analyzed for the following metallic elements by the indicated procedures. Calcium, copper, cobalt, manganese, molybdenum, and zinc were determined by the procedure outlined by Parks *et al.* (1943). The methods recommended by Sandell were used in estimating the cadmium (1939) and lead (1937). Iron was determined by the dipyrldyl method as modified by Kitzes *et al.* (1944). The method outlined by Cholak *et al.* (1943) was used for the estimation of aluminum. The results of these analyses are summarized in table 1.

All fermentations were conducted under anaerobic conditions. Fermentation products were determined by the following methods on the fermented medium. When gases were to be determined, the carbon dioxide was absorbed in alkali and the remaining gas was collected over water and calculated as hydrogen. The carbon dioxide produced was calculated as the difference between that of the fermented culture and that of the uninoculated control flask. Glucose was determined by the method of Shaffer and Somogyi (1933). The glucose values were corrected for acetoin present in the samples. The Virtanen and Pulkki (1928) distillation method was used for the determination of the volatile acids. Lactic acid was extracted from the acidified fermentation liquor with ethyl ether, and determined by the method of Friedemann and Graeser (1933). Succinic acid was also determined on this extract by the method of Phelps *et al.* (1939). The 2,3-butylene glycol was determined by the method of Johnson (1944), and also by the "direct oxidation" method mentioned by Johnson; the latter on a butyl alcohol extract of a portion of the fermented medium saturated with potassium carbonate. After the appropriate acetoin corrections had been applied, the values obtained by these two methods agreed satisfactorily. Ethyl alcohol was determined by the method outlined by Johnson (1932). Corrections were made for the acetoin and 2,3-butylene glycol present in the samples. Acetoin was determined by the method of Langlykke and Peterson (1937).

The following methods were used for the analysis of the fermented media for B vitamins: riboflavin by the Snell and Strong method (1939); pantothenic acid by the procedure of Strong (1941); biotin by the method of Shull *et al.* (1943); nicotinic acid by the procedure of Krehl (1943), and vitamin B<sub>6</sub> activity by the

method outlined by Luckey *et al.* (1944). The samples for the vitamin analyses were hydrolyzed by acid for the riboflavin, biotin, and nicotinic acid assays, and by taka-diastase for the pantothenic acid and B<sub>6</sub> assays, as is recommended by the respective assay methods.

#### EXPERIMENTAL

Several replicate fermentations were set up on each of the media the composition of which is given in table 1. A complete analysis was not made of all the fermentations, but the amounts of solvents and volatile acids formed as well as the glucose fermented were determined in all cases. The analyses presented in table 2 for these fermentations are typical of those obtained on the various media. Recording of the changes in acidity during the fermentation as measured by the

TABLE 2  
*Products of glucose fermentation by A. aerogenes on various media*

	MEDIUM A	MEDIUM B	MEDIUM C
	mM	mM	mM
Glucose fermented . . . . .	10.4	28.6	10.3
Products (per 100 mM fermented glucose):			
Acetic acid . . . . .	12.8	21.3	3.5
Formic acid. . . . .	8.5	27.8	0.01
Lactic acid. . . . .	16.8	7.7	15.5
Succinic acid. . . . .	7.4	10.5	10.2
Ethyl alcohol. . . . .	79.0	81.2	47.8
2,3-butylene glycol. . . . .	42.4	41.8	47.8
Acetoin . . . . .	1.5	1.3	1.2
Carbon dioxide. . . . .	139.0	145.0	163.0
Hydrogen. . . . .	41.6	57.6	91.8
Carbon recovery. . . . .	98.2%	102.0%	98.4%
Oxidation-reduction index. . . . .	1.01	0.96	1.01

change in pH was not possible in those fermentations reported in table 2, since samples could not be taken during the fermentation period. However, samples were taken at various intervals from similar fermentations, and in all cases on the three different media the reaction during the fermentations very rarely went above pH 6.2 and usually ranged between pH 5.6 and 6.0.

From the analyses of these first fermentations it appeared that the distribution of products obtained when medium C was fermented was quite different from that obtained on medium A and medium B. On medium C the quantities of formic acid, acetic acid, and ethyl alcohol were reduced, while the amounts of 2,3-butylene glycol and lactic acid were increased.

Since the only difference between medium B and medium C was the metallic ion content, it seemed possible that the difference in the distribution of fermentation products was due to the metallic ions present in medium B. Accordingly,

various metallic ions were added to medium C in order to test this hypothesis. The amounts of various metallic ions indicated in table 3 were added to medium C, and the fermentations were carried out as before. The results of the analyses for the products of the fermentations are presented in table 3.

TABLE 3

*The effects of added metallic ions on the growth of A. aerogenes on medium C*

A. Effect on fermentation

ION ADDED	QUANTITY	GLUCOSE FERMENTED	DISTRIBUTION OF PRODUCTS*				
			Ethyl alcohol	2,3-butyl- ene glycol	Acetoin	Formic acid	Acetic acid
	$\mu\text{g/L}$	mM	mM	mM	mM	mM	mM
None (flask a).....		18.1	43.0	42.0	1.6	0.1	2.1
(flask b).....		18.7	44.2	41.3	1.4	0.05	4.5
Zinc.....	10	12.6	75.5	43.4	2.1	24.5	10.5
Copper.....	10	11.6	64.0	45.5	2.3	17.0	8.5
Chromium.....	10	15.1	63.9	41.0	1.6	31.0	20.8
Aluminum.....	10	15.6	59.0	45.2	5.0	21.5	6.8
Iron.....	10	15.6	55.8	50.5	1.8	3.2	4.3
Iron.....	20	9.2	60.8	47.8	2.6	18.0	7.5
Manganese.....	10	14.6	60.6	42.8	1.9	16.5	10.5
Manganese.....	20	7.2	86.0	42.0	3.9	28.7	18.1

B. Effect on vitamin synthesis

ION ADDED	QUANTITY	WEIGHT OF CELLS	RIBOFLAVIN	PANTO- THENIC ACID	B <sub>6</sub> †	BIOTIN	NICOTINIC ACID
	$\mu\text{g/L}$	mg/ml	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$
None (flask a).....		0.45	0.18	0.40	4.2	2.7	0.59
(flask b).....		0.47	0.24	0.48	5.0	3.1	0.64
Zinc.....	10	0.60	0.25	0.92	1.6	3.3	0.90
Copper.....	10	0.58	0.18	0.96	2.5	3.5	0.59
Chromium.....	10	0.55	0.26	0.88	2.5	5.3	0.78
Aluminum.....	10	0.66	0.21	1.00	7.5	3.8	0.59
Iron.....	10	0.61	0.20	0.56	2.2	2.6	0.59
Iron.....	20	0.47	0.23	0.84	2.3	3.5	0.57
Manganese.....	10	4762	0.20	0.60	13.5	3.1	0.65
Manganese.....	20	0.53	0.16	0.84	3.7	3.4	0.67

\* Per 100 mm fermented glucose.

† As measured by *S. lactis* R against a standard B<sub>6</sub> solution. This sample of B<sub>6</sub> (crystalline) was obtained from Parke, Davis and Co.

Analysis of the fermented media of the fermentations in table 2 for B vitamins was inadvisable, since at the end of the 48-hour incubation period sufficient phosphoric acid was added to each fermentation to bring it to pH 2. This would probably cause some destruction of the pantothenic acid and the vitamin B<sub>6</sub> present. Those fermentations the analyses of which are presented in table 3 were not made acid at the end of the fermentation period. Instead, aliquots were frozen and later analyzed for the vitamins by the methods previously men-



tioned. In order to obtain the approximate bacterial cell yield, an aliquot of the fermented medium was centrifuged. The precipitated cells were rewashed and recentrifuged several times. The washed cells were dried at 110 C for 8 hours in tared tubes.

Vitamin analyses of the cell-free fermented media were also made. In almost all cases over 90 per cent of the vitamin content of the medium was in the cell-free portion of the medium.

Conditions in the laboratory necessitated limiting the duration of the fermentations to 48 hours. In many cases only part of the carbohydrate had been fermented at the end of this interval. However, since all fermentations were of equal duration, the results should be comparable.

#### DISCUSSION

It can be seen from table 1 that the treatment of such media by passage over a cationic exchange material is successful in reducing the metallic content. It is probable that still further purification could be obtained by passing the treated medium over an anionic exchange material. However, even a single passage over the cationic exchange material as used in these experiments is sufficient to reduce the metallic content of the medium so that studies may be made of the effects of added metallic ions on bacterial metabolism. With this method of purification it is necessary to use highly purified salts in the medium since they cannot be passed through the ionic exchange column, and must be added to the treated sugar solution. It does not seem necessary to use water redistilled several times as the metallic ions present in the water will be removed on passage through the column.

The cation exchange method of treatment has several advantages over methods such as the one used by Waring and Werkman (1943a). The method used by these investigators removes only those metals forming chloroform-soluble or water-insoluble complexes with 8-hydroxyquinoline. The cation exchange method is less specific and will remove any cation present. The passage of sugar solutions over cation exchange materials does not seem to affect the sugar fermentation.

A comparison of the distribution of products after the fermentations of medium B and medium C indicates that several marked changes have taken place. The quantities of volatile acids and ethyl alcohol are much reduced whereas those of lactic acid, 2,3-butyleneglycol, and carbon dioxide are increased.

It appears from the data presented in table 3 that the various enzyme systems governing the dissimilation of glucose by this organism may be activated by several metallic ions. The addition of manganese to medium C in sufficient amounts to bring the level of this metallic ion up to that of medium B caused the fermentation to revert to "normal." The addition of chromium, as well as lesser amounts of manganese, and zinc, copper, and aluminum also caused partial reversion.

The effects of added metallic ions on the B vitamin syntheses, as shown in table 3, vary with each vitamin. The addition of any of the metallic ions with the exception of the higher concentration of iron tended to increase the cell yields.

Considering an increase or decrease of 50 per cent of the vitamin as compared with that formed in the fermented control medium as significant, none of the added metallic ions affected the riboflavin synthesis. On the same basis, all of the added metallic ions stimulated the synthesis of pantothenic acid, especially aluminum. All of the added metallic ions except aluminum and the lower level of manganese were inhibitory to the synthesis of vitamin B<sub>6</sub> (or substances having B<sub>6</sub> activity). The addition of chromium seemed to increase the synthesis of biotin, whereas the others had little or no effect on the synthesis of this vitamin. Zinc seemed to stimulate nicotinic acid synthesis, but the other metallic ions apparently had no effect.

The quantities of B vitamins synthesized by a different strain of *A. aerogenes* under anaerobic conditions as reported by Thompson (1942) are about 25 per cent of those found in these experiments for pantothenic acid, nicotinic acid, and riboflavin. About twice as much biotin and vitamin B<sub>6</sub> (or substances having B<sub>6</sub> activity) were synthesized in his experiments as were found in these investigations. These differences may be due in part to the differences in media and incubation time. He used a medium containing hydrolyzed casein as well as added trace elements, and incubated his culture for 24 hours.

Rodgers (1942) has studied the synthesis of riboflavin by many types of bacteria. When his strain of *A. aerogenes* was cultured under anaerobic conditions, it synthesized 0.08  $\mu$ g of riboflavin per ml of medium. The medium contained glucose, asparagine, and inorganic salts. However, in his experiments only 0.23 mg of cells were produced per ml of medium.

Burkholder and McVeigh (1942) have studied the B vitamin production by a strain of *Bacterium aerogenes*. The medium used for the growth of this organism included glucose, asparagine, tryptophane, cystine, and a trace element solution. The values they report for the synthesis of biotin are about equal to those reported in table 3. They obtained about 20 per cent as much riboflavin and 50 per cent as much nicotinic acid as were found in the experiments summarized in table 3. In their experiments only 15 per cent of the vitamins were found in the cell-free medium.

#### SUMMARY

The preparation of a bacteriological medium low in metallic cations has been accomplished by the passage of the nonionic portion of the medium over a cationic exchange column.

When this purified medium was fermented by *Acrobacter aerogenes*, a redistribution of the fermentation products took place. The quantities of acetic acid, formic acid, and ethyl alcohol formed were decreased, whereas the quantities of lactic acid and carbon dioxide increased.

A reversion to the "normal" fermentation was accomplished by the addition of manganese and chromium ions to the purified medium. The addition of zinc, copper, aluminum, and iron caused a partial reversion.

When the metallic ions were added to the purified medium, they had little effect on the synthesis of riboflavin, biotin, and nicotinic acid by this organism.

The addition of manganese or aluminum at a level of 10  $\mu$ g per liter increased the quantity of vitamin B<sub>6</sub> (or substances having B<sub>6</sub> activity) synthesized, but the same levels of copper, iron, and zinc decreased the synthesis of this vitamin. The addition of zinc, copper, manganese, chromium, or aluminum at a level of 10  $\mu$ g per liter increased the amount of pantothenic acid synthesized.

#### ACKNOWLEDGMENTS

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# ASSIMILATION OF GLUCOSE AND RELATED COMPOUNDS BY GROWING CULTURES OF *PSEUDOMONAS SACCHAROPHILA*

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During the last decade there has been increased interest among microbiologists in assimilation by heterotrophic organisms. With the introduction by Barker (1936), Giesberger (1936), and Clifton (1937) of the Warburg manometric technique for the measurement of assimilation by resting suspensions, a new estimation of the process became possible. The method used for such studies consists of providing a "starved" suspension of organisms with a known amount of a single organic carbon source in the Warburg vessel, and measuring the amounts of oxygen uptake and carbon dioxide production during the period of increased metabolic activity following the addition of substrate. If these amounts are found to be less than those required for complete respiration of the organic compound and no products of incomplete oxidation are present in the medium, it is assumed that the remaining fraction of the compound has been synthesized into cell material. It is to be remembered, however, that the organisms used in such studies are not proliferating, and that their activities may be somewhat different from those of growing cultures. We have as yet almost no real knowledge of the steps involved in the synthesis of protoplasmic material.

The investigations reported here were made in an attempt to compare the assimilation from certain compounds by *Pseudomonas saccharophila* (Doudoroff, 1940) during cell multiplication with the assimilation shown by resting suspensions of the same organism. It is hoped that data on this subject will increase our present understanding at least of the over-all mechanism of synthesis.

*Pseudomonas saccharophila* is a potentially autotrophic hydrogen bacterium, capable of utilizing carbon dioxide as sole carbon source. It grows readily, however, with any of a number of organic compounds as a source of this element, and is strictly aerobic, incapable of fermenting sugars. Except under certain unfavorable conditions to be described, no known metabolic waste products other than CO<sub>2</sub> are formed in heterotrophic metabolism, a fact which makes the organism particularly suitable for the type of studies reported here. Previous investigations (Doudoroff, 1940) using resting suspensions of the organism indicated that its efficiency of assimilation is relatively high. Furthermore, the amount of synthesis which it attains with different carbon sources is remarkably constant and to a certain extent independent of the energy content of the substrate molecule. With glucose, sucrose, maltose, trehalose, lactate, and pyruvate, approximately two-thirds synthesis and one-third oxidation (as measured by oxygen consumption) take place.

## METHODS

The general plan of the present work was to provide the bacterium with a known amount of an organic carbon source and, after a given period of growth,

to determine, by measurement of the amount of organic substrate remaining in the medium, how much had been utilized. From this, the weight of carbon used by the organism could be computed. Then, by carrying out carbon analyses on the bacteria, the percentage of utilized carbon actually appearing in the protoplasm could be found. Thus the figures given in this paper for "per cent synthesis" refer to the percentage of substrate carbon which is assimilated.

The basic medium used for growth of the organisms contained a known amount of an organic carbon source in a mineral medium with the following constituents:  $m/30$   $\text{KH}_2\text{PO}_4$ - $\text{Na}_2\text{HPO}_4$  buffer at desired pH (pH 6.64 was usually employed); 0.1 per cent  $\text{NH}_4\text{Cl}$ ; 0.05 per cent  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.005 per cent  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ; 0.0005 per cent  $\text{CaCl}_2$ . Organic compounds were sterilized by autoclaving in distilled water, with the exception of sodium pyruvate, which was sterilized by filtration. The phosphate buffer, ferric chloride solution, and a mixture of the remaining salts were autoclaved separately in order to avoid precipitation during the application of heat. For growth experiments, the media were placed in Erlenmeyer flasks in amounts such that a large surface was exposed to air (e.g., 30 ml in a 200-ml Erlenmeyer) and the flasks were placed on a rotary shaker to provide constant agitation and ample aeration of the medium during the development of the organisms. Except where otherwise indicated, the temperature of incubation was 30 C. In most cases, it was attempted to remove the flasks toward the end of the logarithmic period of development. When sugars were used as substrates, the amount remaining in the medium after growth was determined according to the method of Hassid (1937), the disaccharides being subjected to a preliminary acid hydrolysis. Acetic acid was measured by steam distillation and titration with standard base. Of the methods tried for estimation of lactic acid, that of Friedemann and Graesser (1933) was most satisfactory. Pyruvic acid determinations were carried out as suggested by Clift and Cook (1932; see also Elliot, Bency, and Baker, 1935). The bacteria on which carbon analyses were to be made were centrifuged, washed once in distilled water acidified with  $\text{KH}_2\text{PO}_4$  to remove carbonates, and then dried on a steam bath. Carbon determinations were carried out either by the dry combustion method described by Pregl (1930) using an  $\text{MnO}_2$ - $\text{PbO}_2$  catalyst or by the wet combustion method of McCready and Hassid (1942), in which the Van Slyke oxidation mixture is used. In both cases, carbon dioxide was determined gravimetrically after absorption. Results with the two methods were found to check fairly well, and both gave 100 per cent recovery when used with known organic compounds.

#### EXPERIMENTAL

*Composition of the bacteria.* Elemental analyses of the bacteria grown in a glucose medium were made by the dry combustion method for carbon and hydrogen and by the Kjeldahl method for nitrogen. The following average values were obtained (on the basis of dry weight): carbon, 54.5 per cent; nitrogen, 11.05 per cent; hydrogen, 7.4 per cent; ash, 3.3 per cent. These figures are fairly comparable with those obtained by Van Niel (1936) for certain purple bacteria. From these figures, the average reduction state of the carbon in the bacterial pro-

toplasm may be computed as approximating the formula ( $\text{CH}_{2.12}\text{O}$ ). The nitrogen content, which is high as compared with that of many tissues, can be decreased very considerably by the removal of the nitrogen source from the medium, as will be shown later.

*Assimilation with different carbon sources.* The average amounts of assimilation obtained from various compounds during growth under optimal conditions, together with the minimal division times observed during the exponential phase of growth at 30 C, are presented in table 1. Obviously, the percentages of synthesis from different compounds by proliferating cells are not at all constant. Furthermore, in all cases the amount of synthesis is less than that found with resting cells (table 1). In experiments with resting cells oxidizing lactic acid or

TABLE 1

*Assimilation of various substrates by resting suspensions and growing cultures of Pseudomonas saccharophila.*

(a) Minimum division time during the phase of exponential (logarithmic) growth at 30 C under optimal conditions (pH, concentration of nutrients) tried.

(b) Percentage of substrate carbon assimilated during growth under optimal conditions.

(c) Percentage of substrate carbon assimilated by resting suspensions on the basis of  $\text{CO}_2$  production (computed from Doudoroff, 1940). Figure for maltose based on corrections discussed previously. Value for pyruvate based on highest values obtained previously and by Bernstein (1944). Acetate value from unpublished data.

(d) Ratio of assimilation during growth to that shown by resting suspensions (b:c).

SUBSTRATE	(a) DIVISION TIME	(b) PER CENT ASSIMI- LATION DURING GROWTH	(c) PER CENT ASSIMI- LATION BY RESTING SUSPENSIONS	(d) RATIO b:c
	min			
Glucose . . . . .	178	54	60	0.90
Maltose . . . . .	170	49	59	0.83
Sucrose . . . . .	109	49	61	0.80
Trehalose . . . . .	105	51	61	0.84
Lactate . . . . .	136	44	61	0.72
Pyruvate . . . . .	152	36	60	0.60
Acetate . . . . .	196	28	45	0.62

glucose, Doudoroff (1940) observed that carbon dioxide is produced in slight excess of the oxygen consumed. This has been taken to indicate the formation of storage products which are, on the average, more reduced than carbohydrates. If it is assumed that the product of primary synthesis is a carbohydrate with the empirical formula  $(\text{CH}_2\text{O})_n$ , the extent of its formation may be computed roughly from the oxygen consumption by respiring cells, as has been done in the previous studies. For comparison with actual synthesis during growth, on the other hand, it was necessary to recompute the amount of assimilation by resting cells from the observed evolution of carbon dioxide, so that these results, too, might be expressed on the basis of the percentage of carbon synthesized. Even with comparable methods of expression, however, it will be seen from the table that growing cultures are less efficient in their over-all synthesis than resting suspensions.



Only with glucose, and to a lesser extent with the disaccharides, does the "growth synthesis" even approach the synthesis by resting suspensions.

The utilization of disaccharides by *P. saccharophila* both in growing cultures and with resting suspensions is somewhat unusual in that sucrose, maltose, and trehalose are all used more rapidly than their constituent hexoses. The rapid utilization of sucrose is particularly striking since fructose is attacked only with very great difficulty. Recent work with enzyme preparations from this organism (Doudoroff, Kaplan, and Hassid, 1943; Doudoroff, 1943) has shown the occurrence of a reversible phosphorolysis of sucrose with the production of glucose-1-phosphate and fructose. As these authors point out, however, this does not explain entirely the behavior of the bacteria with regard to this sugar or throw any light on their action on other disaccharides. With resting suspensions of the bacteria, sucrose and trehalose, though oxidized more rapidly than glucose, are assimilated to about the same extent as this monosaccharide.

The postulation of a two-thirds primary synthesis from pyruvic acid was based on selected experiments using the Warburg technique and depended on the application of certain corrections for autorepiration which were not made with other substrates. In practice, it has been found difficult to obtain closely reproducible results with this substrate, since the previous history of the bacteria, as well as the experimental conditions, appear to affect the efficiency of assimilation from pyruvic acid to a much greater extent than that from sugars. In general, the fraction assimilated was found to be less than two thirds, but, from a large number of experiments, Bernstein (1944) concludes that this figure represents the maximal attainable value for the isolated process of "primary synthesis." It should also be stated that complications are encountered in manometric studies with acetic acid as substrate. Not only does the per cent of assimilation appear to decrease when increasing amounts of this compound are given to the bacteria, but the pH of the medium has a striking effect on the efficiency of synthesis. At high pH values (pH 7.5 to 8.5) the rate of respiration was found to increase and the efficiency of synthesis to decrease progressively with increasing alkalinity. The figures for acetic acid utilization by resting suspensions given in table 1 are based on the average values obtained in experiments conducted in neutral or slightly acid environment. With growing organisms, the amount of assimilation is lower with lactate than with any of the sugars studied, still lower when pyruvate is used as carbon source, and lowest of all with acetate.

*Effect of iron concentration.* The concentration of ferric chloride used in the medium had a pronounced effect on the rate of development of the cultures with all the substrates used. A maximum growth rate was obtained in all cases with about 0.0025 per cent  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ . With lower concentrations, not only was the rate of growth, but also the amount of assimilation, decreased. The effect of iron on both growth rate and assimilation was more pronounced with those carbon sources which gave rapid development (e.g., sucrose) than with substrates utilized more slowly (e.g., glucose). See table 2. With sucrose and trehalose, the iron exerted a striking effect on the course of metabolism during growth. If

insufficient iron was added, pyruvic acid<sup>1</sup> was found to accumulate in the medium, sometimes to such an extent as to result in the death of the organisms. Thus, up to about one third of the sugar was converted into this compound when 0.0001 per cent  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  or less was present. No pyruvic acid, or only traces, could be detected when the concentration was increased to 0.005 per cent. Except in those experiments where the iron concentration was so low that the bacteria appeared to be incapable of further activity after the pyruvic acid accumulation had reached a maximum, this compound was found to disappear more or less rapidly after the depletion of the sugar from the medium, the rate of disappearance depending on the amount of iron available to the organisms. No pyruvic acid was found under any condition in cultures using glucose, maltose, or lactic acid as substrates, nor could its production be shown with suspensions of resting cells grown in iron-deficient media and allowed to oxidize sucrose. That the effect ascribed to iron concentration was not due to impurities present in the

TABLE 2

*Effect of iron concentration on assimilation*

Cultures grown at pH 6.64, 30 C, and harvested just before the exhaustion of substrate

SUBSTRATE	AMOUNT OF $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ INITIALLY ADDED (IN MG PER L)	PERCENTAGE OF SUBSTRATE CARBON ASSIMILATED
M/75 Glucose	5	46
	17	53
	50	53
M/150 Sucrose	5	35
	17	43
	50	48

ferric chloride was shown by the fact that the salt could be replaced by a highly purified solution of ferrous tartrate (tartrate is not oxidized by the bacterium), whereas a mixture of trace elements failed to have the same effect.<sup>2</sup> On the other hand, the amount of iron added to the medium does not truly represent the amount available to the organisms, since during growth of the cultures the colloidal dispersed iron compounds tend to be precipitated out.

*Effect of pH and temperature.* Although assimilation from different substrates by growing cultures shows rather wide variations, the amount from a single substrate is fairly independent of certain changes in environmental conditions. For example, the amount of synthesis during growth with glucose is almost constant between pH 6.2 and 7.4, even though the rate of development is considerably lower in the alkaline range (see table 3). Furthermore, no appreciable difference

<sup>1</sup> Pyruvic acid was precipitated from the medium and identified as the 2,4 dinitrophenyl hydrazone. M.P. (uncorrected): 2,4 dinitrophenyl hydrazone of pyruvic acid, 212.9 C; unknown, 213.0 C; mixed, 213.0 C.

<sup>2</sup> The trace element mixture and ferrous tartrate solution were obtained through the kindness of Dr. D. I. Arnon.

could be detected between glucose cultures grown at room temperature, which showed a synthesis of 52 per cent of the carbon, and those incubated at 30 C.

*Effect of the composition of a'osphere.* Certain alterations of the atmosphere during growth with glucose affected the amount of synthesis rather markedly. As will be seen from table 3, neither the provision of extra carbon dioxide, nor a variation of the oxygen content of the atmosphere between 5 and 20 per cent affected the amount of assimilation. However, removal of all carbon dioxide by its absorption in a cup of alkali within the culture flask resulted in a decreased synthesis. In view of the now well-known role of carbon dioxide in heterotrophic assimilatory processes, this is hardly surprising. The most pronounced effect of

TABLE 3

*Effect of pH, temperature and composition of the atmosphere on assimilation during growth with glucose*

Initial concentration of glucose, M/75 in experiment 1; M/700 in experiment 2. Free access of air allowed in experiment 1; while experiment 2 was carried out in closed flasks. Bacteria were harvested in each case before the exhaustion of either glucose or oxygen.

EXPERIMENT NUMBER	INITIAL COMPOSITION OF ATMOSPHERE	pH	TEMPERATURE	PER CENT OF SUBSTRATE CARBON ASSIMILATED
			C	
1	Air	6.64	20-22	52
	Air	6.2	30	54
	Air	6.64	30	53
	Air	7.4	30	51
2	5% O <sub>2</sub> , 95% N <sub>2</sub>	6.64	30	54
	5% O <sub>2</sub> , 5% CO <sub>2</sub> , 90% N <sub>2</sub>	6.64	30	54
	20% O <sub>2</sub> , 80% N <sub>2</sub>	6.64	30	54
	20% O <sub>2</sub> , 80% N <sub>2</sub> , CO <sub>2</sub> removed*	6.64	30	48
	20% O <sub>2</sub> , 5% CO <sub>2</sub> , 75% N <sub>2</sub>	6.64	30	54
	50% O <sub>2</sub> , 50% N <sub>2</sub>	6.64	30	40

\* CO<sub>2</sub> removed from atmosphere by absorption in KOH placed in a cup within the culture flask. In all other cases, CO<sub>2</sub> was allowed to accumulate in the atmosphere.

a change in atmosphere was observed with high partial pressures of oxygen. Not only did the assimilation decrease as the amount of this gas present was increased up to 50 per cent; it was also observed that growth was entirely absent if as much as 80 per cent oxygen was used. In manometric experiments, resting suspensions were found to assimilate the same percentage of glucose in an atmosphere composed entirely of oxygen as in air. It would therefore appear that the "primary synthetic" mechanism is not affected by an excess of this gas. Furthermore, the autorespiration of resting cells remained the same in an oxygen atmosphere. Apparently, then, oxygen affects some system in the organism connected with growth but not with "primary synthesis."

*Effect of availability of nitrogen source.* Over a limited period of time, the lack of an adequate nitrogen source does not greatly affect the efficiency of assimilation,

as might be expected on the basis of experiments with resting cells, which are provided with no nitrogen source. It will be seen from table 4 that organisms supplied with additional sugar in the absence of ammonium chloride assimilate approximately the same percentage of substrate carbon as when a nitrogen source is present. The composition of the bacteria, however, undergoes a striking change in the absence of nitrogen. A decrease in the nitrogen content of the cells is, of course, to be expected. By analysis of the figures, it can be shown that the total nitrogen in this crop of organisms did not change materially, although there was a small loss, possibly due to experimental error. It will be noted that the amount of sugar used in the same period of time was nearly twice as great in the medium with  $\text{NH}_4\text{Cl}$  as in the nitrogen-free medium. This, of course, was due to the multiplication of the bacteria and the resulting increase in the total

TABLE 4

*Effect of the availability of nitrogen source on assimilation and bacterial composition*

Two aliquots of a culture were centrifuged, washed, and resuspended in medium containing  $\text{m}/150$  glucose, buffer and all usual minerals,\* but in one case without any nitrogen source provided, while in the other with 0.1%  $\text{NH}_4\text{Cl}$ . Original total carbon content of bacteria in each aliquot: 46.6 mg. Incubation at 29 C for 4 hours with constant agitation. Per cent C and N content of bacteria expressed on dry weight basis.

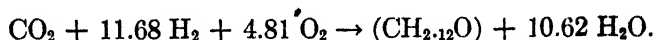
	WITH $\text{NH}_4\text{Cl}$	WITHOUT N SOURCE
mg sugar used . . . . .	222.25	126.2
mg C in sugar used . . . . .	88.9	50.5
mg increase in bacterial C . . . . .	44	22.5
% synthesis . . . . .	49.5	44.7
	%	%
N content of bacteria . . . . .	11.0	7.2
C content of bacteria . . . . .	56	56

\* Note: Only 5 mg of  $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$  per L was added to the suspension, since a large amount of iron was already present in the precipitate which was harvested with the bacteria. The somewhat low amount of assimilation observed in N-containing medium may have been due to an insufficiency of this element.

metabolism of the culture. The efficiency of the assimilation was actually somewhat lower in the nitrogen-free medium. This might seem contrary to expectations, since resting cell suspensions have been found to assimilate even greater quantities of substrate than growing cultures. It must be remembered, however, that the manometric experiments are of very short duration and involve very small amounts of substrate. If the growth experiments had been continued for a longer period of time, the efficiency of assimilation would undoubtedly have shown an eventual decline and would have approached zero, for presumably the synthesis of only nonnitrogenous compounds cannot continue indefinitely.

*Autotrophic development.* As has been mentioned, *P. saccharophila* is a potentially autotrophic bacterium, capable of utilizing carbon dioxide as the sole carbon source in its nutrition. It obtains the energy necessary for the reduction

of carbon dioxide to cell material by the oxidation of gaseous hydrogen to water. In order to study the autotrophic assimilation, a culture of the organism was allowed to develop in the basic mineral medium without any added organic substrate, but in an atmosphere composed initially of 74.5 per cent  $H_2$ , 18 per cent  $O_2$ , and 7.5 per cent  $CO_2$ . Growth was stopped before the partial pressure of any one of the component gases had decreased to half its original value. From the total uptake of gases, and from the amount and reduction state of cell material formed during growth under such conditions, the following over-all formulation for the autotrophic metabolism could be made:



It will be seen that the ratio of oxygen to  $CO_2$  used in the process is approximately 4.81 to 1. This may be considered to be a very inefficient or wasteful use of hydrogen gas, since much lower  $O_2:CO_2$  ratios have been observed with other hydrogen bacteria (Ruhland, 1924). In unpublished studies Doudoroff found that different strains of hydrogen bacteria showed  $O_2:CO_2$  ratios ranging from 2:1 to 5:1 during growth. The ratio appeared to be fairly characteristic of the species or strain and in selected cases was found to be relatively independent of wide variations in the  $O_2:CO_2$  ratio in the atmosphere.

#### DISCUSSION

In agreement with Clifton and Logan's observations (1938) it has been found that, under optimal conditions, at least some substrates (e.g., glucose) are assimilated almost to the same extent on the basis of carbon by growing cultures as by resting cell suspensions. Yet the anabolic end products are very different in the two cases, as is shown by the effect of nitrogen deficiency on cell composition. This suggests that the main losses of substrate carbon and the most wasteful dissipation of energy occurs in the initial stages of substrate breakdown, leading to what is commonly referred to as the "primary synthesis." It would seem that the further transformations of the reserve materials into protoplasm need not involve any further great losses of either the anabolized carbon or of energy. The discrepancies in assimilation between resting and growing cells with pyruvate and particularly with acetate as substrates would suggest, on the other hand, that the formation of protoplasm does not proceed entirely from the products of primary synthesis but requires also the participation of the substrate.

The effect of iron concentration on the amount of synthesis is by no means clear. Only with sucrose and trehalose as substrates was a deficiency of this element observed to have a marked influence on the course of metabolism. In iron-deficient media with these sugars, pyruvic acid appeared in large quantities during growth, but not in experiments with resting cells. Bernstein (1944) showed that the iron concentration in the medium, together with the developmental state of the culture, had a marked effect on the course of oxidation of dicarboxylic acids by the same organism. It is interesting that in his experiments pyruvic acid was found to accumulate only in resting cell suspensions and not in the growing cultures, and only when sufficient iron was present.

Pyruvic acid has also been shown to accumulate in the medium when *P. saccharophila* oxidizes glucose in the presence of dinitrophenol (Doudoroff, 1940). In suitable concentrations, this poison appears to inhibit assimilation without affecting materially the rate of oxygen uptake. It seems likely that the iron deficiency has a similar effect on the oxidation of sucrose and trehalose in that the later stages of oxidation, involving pyruvic acid, cannot keep pace with the production of this compound. Whether this is brought about primarily by a suppression of assimilation or by the overloading of the hemin system can only be a matter of speculation, since the two possibilities cannot at present be investigated separately. Bernstein's results can also be explained by assuming that the hemin system is affected by the availability of iron during growth, but that under the conditions of his experiment the rate of oxidation of fumarate to pyruvate and  $\text{CO}_2$  is more dependent on iron concentration than are the further oxidations involving pyruvate. It is conceivable that, although the mechanism of "primary synthesis" is the same with disaccharides as with hexoses, some later process necessary to growth cannot quite keep up with the rapid assimilation of disaccharides. This would result in a loss of efficiency and account for the somewhat lower values for assimilation obtained with cultures growing with the disaccharides. Furthermore, with lactic and pyruvic acids which support rapid development, synthesis during growth under optimal conditions is even lower, although assimilation by resting cells is almost as great as with glucose (see table 1). The effect of iron deficiency may then be to accentuate the discrepancy between catabolic and anabolic processes. This would explain the higher iron requirements for maximum efficiency of assimilation with the disaccharides than with glucose.

#### SUMMARY

In a study of the assimilation with different substrates by growing cultures of *Pseudomonas saccharophila*, it has been shown that:

1. Whereas synthesis during growth with some substrates (glucose) is not greatly different from the "primary synthesis" shown by resting cell suspensions, with other substrates (lactate, pyruvate, and acetate) a rather great discrepancy appears between the results obtained in the two types of studies.
2. With glucose as substrate, moderate variation in the temperature of incubation, the composition of the atmosphere, and the pH of the medium have no appreciable effect on the efficiency of assimilation.
3. Complete removal of  $\text{CO}_2$  decreases synthesis, as do high concentrations of oxygen. With very high partial pressures of oxygen no growth occurs, although no effect of such atmospheres can be observed on the behavior of resting cell suspensions.
4. An insufficiency of nitrogen does not materially affect the percentage of synthesis over a limited period of time, although it brings about a striking alteration in the composition of the organisms.
5. An insufficiency of iron in the medium results in a decreased synthesis, particularly striking with those substrates utilized more rapidly than glucose.

Pyruvic acid accumulates in iron-deficient media with sucrose and trehalose, but not with the other substrates studied as carbon sources.

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# A SEROLOGICAL STUDY OF STRAINS OF *ALCALIGENES RADIOBACTER* AND *PHYTOMONAS TUMEFACIENS* IN THE "M" AND "S" PHASES<sup>1</sup>

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The cultural and morphological similarity of *Phytomonas tumefaciens* and *Alcaligenes radiobacter* has been recognized for many years. Many attempts have been made to devise more adequate means of distinguishing cultures of these two forms without resorting to differentiation on the basis of plant infection studies (Conn, Wolfe, and Ford, 1940). To a lesser extent there has been difficulty in distinguishing strains of these two species from certain species of the genus *Rhizobium* (Hofer, 1941). The results obtained in these attempts at laboratory differentiation have served to emphasize the similarity of these forms in laboratory media and to place the final burden of identification upon the ability to infect a susceptible host (Hendrickson, Baldwin, and Riker, 1934).

Inasmuch as the serological studies of Reid, Naghski, Farrell, and Haley (1942) have shown that several plant pathogens characterized as species of *Phytomonas* are in fact transitory adaptations of *Pseudomonas fluorescens*, and the studies of Elrod and Braun (1942) have revealed a similar relationship between certain other forms characterized as *Phytomonas* species and *Pseudomonas aeruginosa*, an examination of *Phytomonas tumefaciens* and *Alcaligenes radiobacter* was undertaken in an effort to determine the relationship of these two similar organisms.

## METHODS

*Cultures used.* The cultures of *Phytomonas tumefaciens* and *Alcaligenes radiobacter* used in this study were obtained from the stock culture collection of the Division of Bacteriology, New York State Agricultural Experiment Station, Geneva, N.Y., through the courtesy of Dr. A. W. Hofer. These strains are ones with which many research workers in the fields of soil bacteriology and plant pathology are acquainted and have been employed by a number of these workers in their investigations (Hendrickson, Baldwin, and Riker, 1934; Hofer, 1941). The following seven strains of *Alcaligenes radiobacter* were used.

R1-1a. Isolated by W. H. Wright of the University of Wisconsin in 1924.

R3. Isolated by E. B. Fred of the University of Wisconsin in 1927.

R3sc1. Single cell isolation from culture R3.

S36. Isolated by Löhnis and Beijerinck in 1904.

S192. Isolated by N. R. Smith of the U. S. D. A. in 1927.

1000. An isolation made by F. Löhnis, date unknown.

ISC. Isolated by R. Hansen of the University of Illinois in 1923.

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The strains of *Phytomonas tumefaciens* used were ScT5fff1 and ScA-1. These are single cell isolates made at the University of Wisconsin. The two strains of *Phytomonas tumefaciens* and six of the seven strains of *Alcaligenes radiobacter* are known as typical of their respective species. Strain S36 of *Alcaligenes radiobacter*, however, has been described by workers in other laboratories as "atypical."

The strains of the two species were tested on tomato plants to check pathogenicity prior to use in this study. The tests were conducted with the organisms in the Dawson "M" phase as received at this laboratory. The strains of *Phytomonas tumefaciens* proved virulent for the tomato plant and the strains of *Alcaligenes radiobacter* proved noninfective.

**Media employed.** Stock cultures of the organisms in the Dawson "M" phase were held on yeast-extract mannitol mineral-salts agar. In decapsulation attempts yeast-extract mineral-salts broth was used and similar mannitol-free media were employed in the culture of the organisms in the Dawson "S" phase.

**Serological techniques.** Live antigen suspensions of 48-hour cultures were used in rapid immunization of rabbits. Daily doses of 1.0 ml were given following an initial dose of 0.1 ml and injections continued until approximately 16 ml had been administered intravenously. Titers were determined by the use of standard macroscopic agglutination tests.

Conversion of the Dawson "M" to the Dawson "S" phase of the organism was accomplished by daily transfer in yeast-extract mineral-salts broth containing 10 per cent of the homologous antiserum. The conversion from "M" to "S" phase was observed by daily streaking on solid media, and serological examinations of isolates from the streak plates were made in the case of colonies which exhibited "S" characteristics, according to the method of Dawson and Sia (1931).

Agglutinin absorption tests were performed in accordance with the method of Krumwiede, Cooper, and Provost (1925).

## RESULTS

Three of the "typical" strains of *Alcaligenes radiobacter*, R1-1a, R3, and R3sc1, and the two strains of *Phytomonas tumefaciens*, ScT5fff1 and ScA-1, were selected for use as antigens in the "M" phase for animal immunization. The titers obtained with strains R3sc1 and ScT5fff1 are shown in tables 1 and 2, respectively. Titers obtained with antisera from animals immunized with strains R1-1a and R3 were similar to those obtained with R3sc1, and antiserum obtained from an animal immunized with ScA-1 proved similar to that obtained from the use of ScT5fff1, and consequently the results obtained are not shown in tabular form.

As shown in table 1, the six typical strains of *Alcaligenes radiobacter* proved to be similar in antigenic composition in the "M" phase. The atypical strain S36, on the other hand, proved serologically unlike the other six in the mucoid state. The serological examination effected a separation of this strain from the typical strains similar to that made in other laboratories on the basis of cultural tests. It is also to be noted from the data presented in table 1 that the strains of *Phytomonas tumefaciens* employed are antigenically unlike the typical strains of *Alcaligenes radiobacter* in the Dawson "M" phase and no cross agglutination occurred in significant dilutions.

The antiserum prepared with the use of *Phytomonas tumefaciens* ScT5fff1 proved almost equally effective in the agglutination of cells of the other strain of *Phytomonas tumefaciens*, as shown in table 2. As would be expected from the results with antiserum prepared against *Alcaligenes radiobacter* "M" phase cells (table 1), no agglutination of *Alcaligenes radiobacter* cells occurred in the presence of the *Phytomonas tumefaciens* "M" phase antiserum.

Since in each case typical strains within the species proved antigenically similar in the "M" phase, the strains which had been employed as antigens for animal immunization in the "M" phase were selected for further investigation in the

TABLE 1

Agglutination titers of antiserum prepared against *Alcaligenes radiobacter* R3sc1  
Dawson "M" phase

CELLS	DILUTION									CON- TROL
	50	100	200	400	800	1,600	3,200	6,400	12,800	
<i>A. radiobacter</i> R1-1a.....	3+	3+	4+	4+	4+	4+	4+	3+	+	—
<i>A. radiobacter</i> R3.....	3+	3+	3+	4+	4+	4+	4+	3+	+	—
<i>A. radiobacter</i> R3sc1.....	3+	3+	4+	4+	4+	4+	4+	2+	+	—
<i>A. radiobacter</i> S36.....	—	—	—	—	—	—	—	—	—	—
<i>A. radiobacter</i> S192.....	2+	2+	4+	4+	3+	2+	2+	+	±	—
<i>A. radiobacter</i> 1000.....	4+	4+	4+	4+	4+	4+	4+	2+	+	—
<i>A. radiobacter</i> ISC.....	2+	2+	3+	4+	4+	4+	3+	2+	+	—
<i>P. tumefaciens</i> ScT5fff1.....	±	—	—	—	—	—	—	—	—	—
<i>P. tumefaciens</i> ScA-1.....	+	—	—	—	—	—	—	—	—	—

TABLE 2

Agglutination titers of antiserum prepared against *Phytomonas tumefaciens* ScT5fff1  
Dawson "M" phase

CELLS	DILUTION									CON- TROL
	40	80	160	320	640	1,280	2,560	5,120	10,240	
<i>A. radiobacter</i> R1-1a . . . . .	±	—	—	—	—	—	—	—	—	—
<i>A. radiobacter</i> R3 . . . . .	±	—	—	—	—	—	—	—	—	—
<i>A. radiobacter</i> R3sc1 . . . . .	±	—	—	—	—	—	—	—	—	—
<i>P. tumefaciens</i> ScT5fff1 . . . . .	3+	4+	4+	3+	4+	4+	4+	4+	2+	—
<i>P. tumefaciens</i> ScA-1 . . . . .	2+	2+	3+	3+	3+	3+	2+	2+	2+	—

Dawson "S" phase. Removal of the capsule from the cells of *Alcaligenes radiobacter* R3sc1 was first attempted and was accomplished with 19 successive transfers in yeast-extract mineral-salts broth containing 10 per cent homologous antiserum. Decapsulation of *Phytomonas tumefaciens* ScT5fff1 was next attempted and was accomplished with little difficulty in 7 successive transfers. Likewise, the removal of capsular material from cells of *Phytomonas tumefaciens* ScA-1 did not prove difficult.

With much smaller inocula than those employed in previous experiments, cells of *Alcaligenes radiobacter* R1-1a and R3 were freed of capsular material in 12 and

11 successive transfers, respectively. The relative stability of the "M" phase of *Alcaligenes radiobacter* strains as contrasted with the "M" phase of *Phytomonas tumefaciens* strains was obvious in all attempts at conversion.

TABLE 3  
Agglutination titers of antiserum prepared against *Alcaligenes radiobacter* R3sc1  
Dawson "S" Phase

CELLS	DILUTION									CON- TROL
	40	80	160	320	640	1,280	2,560	5,120	10,240	
<i>A. radiobacter</i> R3sc1 "S" .....	2+	3+	4+	4+	4+	4+	4+	2+	—	—
<i>A. radiobacter</i> R3sc1 "M" .....	±	—	—	—	—	—	—	—	—	—
<i>A. radiobacter</i> R1-1a "M" .....	±	—	—	—	—	—	—	—	—	—
<i>A. radiobacter</i> R3 "M" .....	±	—	—	—	—	—	—	—	—	—
<i>P. tumefaciens</i> ScT5fff1 "S" .....	2+	2+	4+	4+	4+	4+	3+	2+	—	—
<i>P. tumefaciens</i> ScT5fff1 "M" .....	±	—	—	—	—	—	—	—	—	—
<i>P. tumefaciens</i> ScA-1 "M" .....	±	—	—	—	—	—	—	—	—	—

TABLE 4  
Agglutination titers of antiserum prepared against *Phytomonas tumefaciens* ScT5fff1  
Dawson "S" phase

CELLS	DILUTION									CON- TROL
	40	80	160	320	640	1,280	2,560	5,120	10,240	
<i>A. radiobacter</i> R3sc1 "S" .....	2+	3+	4+	4+	3+	3+	4+	2+	—	—
<i>A. radiobacter</i> R3sc1 "M" .....	±	—	—	—	—	—	—	—	—	—
<i>A. radiobacter</i> R1-1a "M" .....	±	—	—	—	—	—	—	—	—	—
<i>A. radiobacter</i> R3 "M" .....	±	—	—	—	—	—	—	—	—	—
<i>P. tumefaciens</i> ScT5fff1 "S" .....	2+	2+	2+	3+	3+	3+	3+	2+	—	—
<i>P. tumefaciens</i> ScT5fff1 "M" .....	±	—	—	—	—	—	—	—	—	—
<i>P. tumefaciens</i> ScA1 "M" .....	±	—	—	—	—	—	—	—	—	—

TABLE 5  
Agglutination titers of antiserum prepared against *Alcaligenes radiobacter* R3sc1  
Dawson "M" phase

CELLS	DILUTION									CON- TROL
	40	80	160	320	640	1,280	2,560	5,120	10,240	
<i>A. radiobacter</i> R3sc1 "S" .....	—	—	—	—	—	—	—	—	—	—
<i>A. radiobacter</i> R3sc1 "M" .....	4+	4+	4+	4+	4+	4+	4+	4+	3+	—
<i>P. tumefaciens</i> ScT5fff1 "S" .....	—	—	—	—	—	—	—	—	—	—
<i>P. tumefaciens</i> ScT5fff1 "M" .....	—	—	—	—	—	—	—	—	—	—

Following conversion of the three strains of *Alcaligenes radiobacter* and the two strains of *Phytomonas tumefaciens* from the "M" to "S" phase, rabbits were rapidly immunized with these cultures in the "S" phase. Agglutination reactions

obtained with antisera prepared with the use of *Alcaligenes radiobacter* R3sc1 and *Phytomonas tumefaciens* ScT5fff1 are shown in tables 3 and 4. The reactions obtained with antisera prepared against the other three strains did not differ from those shown in these tables.

The data presented in tables 3 and 4 show that the antisera prepared against these strains in the "S" phase fail to agglutinate the "M" phase cells of the homologous strains but, on the other hand, indicate a striking serological similarity between the "S" phase cells of the two species.

Tests of "S" phase cells against antisera prepared with "M" phase organisms are shown in tables 5 and 6. It is to be noted that conversion to the "S" phase rendered these cells inagglutinable by antiserum prepared against the homologous strains in the "M" phase.

The antigenic similarity of the "S" phase cells of the two species as shown in tables 3 and 4 was made the subject of further study. The agglutinin absorption technique was employed in an examination of the "S" phase cells and the antisera prepared with the use of such cells. Complete reciprocal agglutinin absorption

TABLE 6

*Agglutination titers of antiserum prepared against Phytomonas tumefaciens ScT5fff1  
Dawson "M" phase*

CELLS	DILUTIONS									CON- TROL
	40	80	160	320	640	1,280	2,560	5,120	10,240	
<i>P. tumefaciens</i> ScT5fff1 "S".....	—	—	—	—	—	—	—	—	—	—
<i>P. tumefaciens</i> ScT5fff1 "M".....	3+	3+	4+	4+	3+	4+	4+	4+	2+	—
<i>A. radiobacter</i> R3sc1 "M".....	—	—	—	—	—	—	—	—	—	—
<i>A. radiobacter</i> R3sc1 "S".....	—	—	—	—	—	—	—	—	—	—

was found by the method of Krumwiede, Cooper, and Provost (1925) in the examination of these five strains and respective antisera, indicating that in the "S" phase these strains of *Alcaligenes radiobacter* and *Phytomonas tumefaciens* are serologically identical.

#### DISCUSSION

The results obtained in the serological study of typical strains of *Alcaligenes radiobacter* and *Phytomonas tumefaciens* in the Dawson "M" phase are in agreement with the findings of Riker, Banfield, Wright, Keitt, and Sagen (1930). These workers likewise found no serological similarity between representatives of these two species in the "M" phase.

A similar apparent lack of serological relationship in the "M" phase was shown by Harris (1940) to exist within the species *Pseudomonas aeruginosa*. Decapsulation of these dissimilar strains of *P. aeruginosa* by Harris, however, yielded "S" phase cells which, by the agglutinin absorption technique, were shown to be antigenically identical. The use of decapsulation and agglutinin absorption techniques in this study have yielded results comparable to those obtained by

Harris in his studies of strains of *Pseudomonas aeruginosa*. Although the strains of *Alcaligenes radiobacter* and *Phytomonas tumefaciens* used showed no serological similarity in the "M" phase, this difference proved to exist only in the "M" phase, whereas antigenic identity was found to exist in the "S" phase.

These results suggest that the organisms recognized as *Alcaligenes radiobacter* and *Phytomonas tumefaciens* comprise a single species and in their usual "M" phase bear the same relation to each other as do the types of the pneumococcus to one another.

#### SUMMARY

An investigation has been made of the serological relationship of *Alcaligenes radiobacter* and *Phytomonas tumefaciens* with the use of agglutination and agglutinin absorption techniques employed in the study of typical strains in the Dawson "M" and "S" phases.

It has been found that in the strains under study *Alcaligenes radiobacter* and *Phytomonas tumefaciens* differ serologically in the "M" phase. The same strains are antigenically identical in the "S" phase, as indicated by complete reciprocal agglutinin absorption.

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# SULFAMETHAZINE: IN VITRO ACTION ON ENTERIC PATHOGENS AS COMPARED WITH SULFADIAZINE AND SULFAMERAZINE<sup>1</sup>

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New sulfonamides are usually tested against bacteria or diseases wherein the older drugs work satisfactorily. There seems to be little advantage in testing new drugs *only* against those bacteria in which older sulfonamides have already proved their worth. Since the older sulfonamides exert little action on diseases caused by some gram-negative organisms, it seemed advisable to attempt to find one which might prove efficacious in treatment of typhoid and paratyphoid infections. Four sulfonamides which were tested previously (Schweinburg and Yetwin, 1944) were not satisfactory so we decided to investigate the potentialities of sulfamethazine.

We do not overestimate conclusions as to clinical application that may be drawn from test tube experiments. Although some believe there is no quantitative relationship between *in vitro* and *in vivo* action (Marshall, Litchfield, White, Bratton, and Shepherd, 1942), other recent papers reveal a surprising parallelism between resistance *in vitro* and unsuccessful treatment (Cohn, Steer, and Seijo, 1942; Spink and Vivino, 1944; Winchester and Whittle, 1944). If a drug is an effective bactericidal and bacteriostatic agent in the test tube, it may or may not be effective *in vivo*. But it cannot be expected that a drug ineffective against a certain bacterium *in vitro* will prove successful in combating a disease caused by that organism.

Two new sulfa drugs, sulfapyrazine and sulfamethazine, are now in use, both apparently effective against gram-positive and gram-negative cocci. At the present time the output of sulfapyrazine is apparently limited and we therefore regret our inability to obtain it for examination upon the enteric organisms. The other sulfonamide, sulfamethazine (called sulphamezathine by the English), is dimethylsulfadiazine and consequently not only related to sulfadiazine but also to sulfamerazine, which is monomethylsulfadiazine. The *in vitro* action of sulfamethazine closely resembles that of sulfapyridine when tested against streptococci and pneumococci (Rose, Martin, and Bevan, 1943). Clinical trials with both drugs have shown good results in dysentery but no effect against the *Salmonella* infections (Hardy and Watt, 1944). Sulfamethazine is probably the best of all sulfonamides in the treatment of pneumonia (Page, 1944); it apparently has some advantages over the other sulfonamides now in use (Rose, Martin,

<sup>1</sup> Sharp and Dohme, Incorporated, of Philadelphia furnished the drugs used in these studies.

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and Bevan, 1943). Successful therapeutic application has also been reported in cases of bronchopneumonia, pneumococcic meningitis, streptococcic and meningococcic diseases, coli cysto-pyelitis, and in *Shigella* infections in which other sulfonamides are equally effective (Jennings and Patterson, 1942; Pakenham-Walsh, 1943; Hardy and Watt, 1944; Page, 1944).

Some investigators have shown that if one sulfonamide proves effective against a particular bacterium, the others will follow suit, although there may be slight variations of the necessary concentrations. Conversely, if one sulfonamide is ineffective against a certain organism, the others will also prove ineffective (Wyss, Grubagh, and Schmelkes, 1942). Although we are in accord with this opinion, we found, in the *test tube*, marked differences in the effective concentrations of various sulfonamides on different enteric pathogens (Schweinburg and Yetwin, 1944).

#### MATERIALS AND TECHNIQUE

The three drugs compared (sulfamethazine, sulfadiazine, and sulfamerazine) were dissolved in sterile nutrient broth containing 0.5 per cent peptone. Clear solutions were obtained in concentrations as high as 2.0 per cent with a pH of 7.6, using heat for complete dissolution. The 2.0 per cent solution of sulfamerazine contained small amounts of undissolved powder at room temperature, making gentle heating necessary prior to use. Sulfadiazine and sulfamethazine remained in complete solution at the 2.0 per cent strength. All lower drug concentrations were prepared by diluting these stock solutions with appropriate amounts of sterile broth of the same pH. The concentrations used were: 2.0, 1.5, 1.0, 0.75, 0.50, 0.25, 0.10, 0.050, 0.025, and 0.010 per cent. The following bacteria were examined: *Escherichia coli*, *Eberthella typhosa*, *Salmonella paratyphi*, *Salmonella schottmuelleri*, *Salmonella* var. *enteritidis*, *Salmonella choleraesuis*, *Shigella dysenteriae*, *Shigella paradysenteriae* var. Flexner, *Shigella paradysenteriae* var. Hiss, and *Shigella sonnei*.

Twenty-four-hour cultures were prepared on agar slants. A loopful of the growth was transferred into 5 ml of nutrient broth (pH 7.6) and incubated for 24 hours. Dilutions of this growth were made in isotonic saline and standardized against a nephelometer so that each bacterial suspension contained 16 million bacteria per ml. We found that the same nephelometer could be used for different bacteria without making an appreciable error. We ascertained by plating out that the same degree of cloudiness in tubes containing the various bacteria always meant approximately the same number of organisms. The standardized suspensions were diluted 1:100 with saline, and 0.1 ml (equivalent to 16,000 bacteria) was added to 0.9 ml of the respective drug concentrations, and also to a tube of 0.9 ml nutrient broth serving as control. The number of bacteria chosen was an arbitrary one. The effect of drug concentration on bacteria seems to vary with the size of the inoculum (Pike and Acton, 1942)—a problem we will deal with in detail in a paper to follow. After 24 hours the tubes were read for degree of cloudiness, and 0.1 ml of suitable dilutions was mixed with 10 ml of melted agar, plated, and counted after another 24 hours of incubation. The duration

of contact may influence the action of the drug on bacteria—a factor which will also be considered in the next report.

Following the procedure outlined, we determined the bactericidal action of the three drugs on each of the bacteria examined. We also established the lowest concentration at which bacteriostasis still occurred by comparing the number of bacteria grown in lower drug concentrations with the number found in the broth control. Each drug was tested against the individual bacterium at least 10 times. In a few instances in which the results were not uniform from the very beginning, as many as 20 experiments were performed; but in no case did the bactericidal or bacteriostatic titer deviate for more than one tube from the final result. The following protocol (table 1) is an example of the procedure followed in each of the 340 tests.

TABLE 1  
Sample protocol

Organism: *Escherichia coli*

Protocol: 44

Drug: Sulfamethazine

Tubes inoculated: 2/8/44

Plates made: 2/9/44

TUBE	CONCENTRATION OF DRUG	CLOUDINESS OF TUBES	DILUTION USED	TOTAL NUMBER BACTERIA RECOVERED
1	2.0%	Clear	0	0
2	1.5%	Clear	0	0
3	1.0%	Clear	0	0
4	0.75%	Trace (?)	0	0
5	0.50%	Trace	1:30,000	1.5 million
6	0.25%	Trace +	1:1 million	250 million
7	0.10%	Moderate	1:1 million	1,200 million
8	0.050%	Moderate +	1:10 million	2,200 million
9	0.025%	Heavy	1:10 million	5,000 million
10	0.010%	Heavy	1:10 million	5,000 million
11	None (control)	Heavy	1:10 million	5,000 million

#### RESULTS AND DISCUSSION

The results obtained from our trials are listed in table 2. They are valid for the number of bacteria we employed, for the applied time, for the particular strain of each species tested, and for the medium used. We are well aware, however, that all conclusions drawn from these test tube experiments are of limited value as far as the action of sulfonamides in a diseased organism is concerned. The results show that 9 of the 10 bacteria tested are destroyed in lower concentrations of sulfamethazine than of sulfadiazine or sulfamerazine. The only exception is *Shigella dysenteriae*, in which both sulfadiazine and sulfamerazine act better, the latter being by far the most effective. In 7 of the 10 organisms, sulfamethazine also exhibits the best bacteriostatic effect. Of the remaining three organisms, the Flexner strain is equally affected by sulfamethazine and sulfamerazine, sulfadiazine being least effective. Against *Eberthella typhosa*, sulfadia-



zine works better than sulfamethazine as a bacteriostatic agent; and against *Salmonella* var. *enteritidis*, sulfamerazine was more effective in bacteriostatic range than sulfamethazine.

Compared with the phthalyl derivatives reported upon earlier (Schweinburg and Yetwin, 1944), it is seen that the *in vitro* bactericidal action of sulfamethazine

TABLE 2  
*Bactericidal and bacteriostatic titer of sulfonamides on various intestinal bacteria*

ORGANISM	ACTION	SULFA-DIAZINE	SULFA-MERAZINE	SULFA-METHAZINE
		%	%	%
<i>Escherichia coli</i>	A	1.5	2.0	0.75
	B	0.10	0.10	0.050
<i>Eberthella typhosa</i>	A	1.5	2.0	0.50
	B	0.10	1.0	0.25
<i>Salmonella paratyphi</i>	A	1.0	2.0+	0.75
	B	0.50	1.0	0.10
<i>S. schottmuelleri</i>	A	1.5	2.0	1.0
	B	0.50	1.0	0.25
<i>S. var. enteritidis</i>	A	1.5	1.5	1.0
	B	0.50	0.10	0.25
<i>S. choleraesuis</i>	A	1.5	2.0+	0.75
	B	1.0	0.50	0.25
<i>Shigella dysenteriae</i>	A	0.50	0.10	0.75
	B	0.050	0.050	0.010
<i>Shig. paradysenteriae</i> var. Flexner	A	1.0	1.5	0.75
	B	0.50	0.050	0.050
<i>Shig. paradysenteriae</i> var. Hiss	A	2.0	2.0	0.50
	B	1.0	1.0	0.10
<i>Shigella sonnei</i>	A	2.0	1.5	0.75
	B	0.50	0.50	0.25

A—bactericidal concentration (complete destruction of bacteria).

B—bacteriostatic concentration (partial inhibition of growth as compared with control tube).

is markedly superior against all bacteria tested except the Shiga and Flexner strains. Its superiority is most marked against *Salmonella paratyphi*. It is also more effective in the bacteriostatic range against all the bacteria tested except *Salmonella* var. *enteritidis* and the Flexner strain. Its superiority is outstanding against the two paratyphoid strains and the Hiss strain.

## SUMMARY

Sulfamethazine *in vitro* is by far a more effective bactericidal and bacteriostatic agent than the other sulfonamides examined against *Eberthella typhosa*, *Escherichia coli*, and the *Salmonella* varieties. A similar conclusion may be drawn in its use against *Shigella sonnei* and the Hiss dysentery strain. The drug is effective in the test tube on the typhoid-colon group in concentrations which would suggest that clinical application should be attempted, especially since all other sulfonamides offer no convincing evidence of their ability to combat cases of typhoid fever or of its chronic carrier state (Long, 1941; Cutting and Robson, 1942; Kirby and Rantz, 1942) and of the *Salmonella* infections (Bornstein and Strauss, 1941; Bornstein, 1943).

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# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## EASTERN NEW YORK BRANCH

ALBANY, NEW YORK, DECEMBER 8, 1944

**CARDIOLIPIN—A NEW PHOSPHOLIPID.** *Mary C. Pangborn*, Division of Laboratories and Research, New York State Department of Health, Albany.

The preparation and properties of cardiolipin were briefly reviewed (J. Biol. Chem., **143**, 247; **153**, 343). Chemically, the substance may be described as a complex phosphatidic acid. On alkaline hydrolysis it yields unsaturated fatty acids, chiefly linoleic, and a water-soluble phosphoric acid that breaks down on further hydrolysis with acids into glycerophosphoric acid and glycerol. The original water-soluble phosphorous compound is labile and difficult to characterize satisfactorily, and the possibility of the presence of phosphoric acids other than glycerophosphoric cannot yet be excluded.

**CARDIOLIPIN IN MACRO- AND MICROPRECIPITATION TESTS FOR SYPHILIS.** *Rachel Brown*, Division of Laboratories and Research, New York State Department of Health, Albany.

The preliminary studies dealing with the standardization of antigens composed of the purified substances, cardiolipin, lecithin, and cholesterol, to be used for the serodiagnosis of syphilis by precipitation, have included both macro- and micro-procedures. Present data suggest that the same proportion of lecithin and cardiolipin, about 26:1, is optimum for antigens for both tests but that the proportion of cholesterol is greater in the antigen for the micro-test; the actual amount of the antigen constituents is less in the micro-test. When known syphilitic sera, undiluted and diluted, were examined by both tests, two kinds of typical reactions occurred: (1) The results in the two tests were similar and corresponded to those in complement-fixation tests. (2) The micro-test showed a higher degree of reactivity than the macro-test, and corresponded more closely to the reac-

tion in the complement-fixation test. Thus, as adjusted at present, the microprecipitation test with the purified antigen appears to be more sensitive in some cases of syphilis than the macro-test.

**THE STANDARDIZATION OF CARDIOLIPIN-LECITHIN-CHOLESTEROL ANTIGEN IN THE COMPLEMENT-FIXATION TEST FOR SYPHILIS.** *Elizabeth Maltaner and Frank Maltaner*, Division of Laboratories and Research, New York State Department of Health, Albany.

Relationships between the three chemically pure constituents—cardiolipin, lecithin, and cholesterol—that are essential to an accurate titration of serologic reactivity by complement fixation were determined by a standard technique. Cholesterolized and noncholesterolized solutions were dispersed by rapid and slow admixture respectively with salt solution. The marked inhibitive reaction of cardiolipin for complement was completely obscured by 5 parts of lecithin or by “negative” or moderately reacting serum in the proportion used in tests; strongly reacting sera definitely increased its inhibitive effect. Lecithin was not anticomplementary; it reacted slightly with 1 of 15 specimens of high titer. Cholesterol alone was inert. Mixtures of lecithin and cardiolipin had marked antigenic reaction, which was maximum when the relative proportion was 5:1. Cholesterol increased the reactivity of such mixtures to a maximum when present in the ratio of 3.4:1 with lecithin. The optimally reactive saline dilutions of antigens were directly proportional to their concentration in alcohol. Somewhat greater reactivity was obtained by decreasing the proportion of lecithin and increasing that of cholesterol, but the most reactive of such mixtures were anticomplementary. A ratio of lecithin:cardiolipin of 5:1 and of cholesterol:lecithin of 3.4:1 is considered a con-

servative adjustment for use in complement-fixation tests.

**THE IN VITRO EFFECTS OF N-SUBSTITUTED *p*-AMINOBENZOIC ACID DERIVATIVES UPON SULFONAMIDES WITH PARTICULAR REFERENCE TO LOCAL ANESTHETICS.** *G. R. Goetchius*, Research Laboratories, Winthrop Chemical Company, Inc., Rensselaer, N. Y.

It was found that procaine hydrochloride (U.S.P.), a local anesthetic derived from *p*-aminobenzoic acid, exerted an antagonistic activity toward the sulfonamides almost equal to that of *p*-aminobenzoic acid itself. An antagonistic reaction toward the antibacterial actions of sulfonamides was not displayed by tetracaine hydrochloride (U.S.P.), a local anesthetic derived from *p*-butylaminobenzoic acid. This raised the question as to whether N-substitution in general affected the antisulfonamide action of *p*-aminobenzoic acid and its derivatives. Accordingly, a series of *p*-aminobenzoic acid derivatives was prepared in which alkyl and acetyl groups were substituted in the N-position. The substitutions either greatly reduced or completely nullified the antagonism of the compounds to the sulfonamides when tested against organisms susceptible to sulfonamide action.

**THE ANTIBACTERIAL EFFECTS OF *p*-AMINO-ALKYLBENZENESULFONAMIDES.** *C. A. Lawrence*, Winthrop Chemical Company, Inc., Rensselaer, N. Y.

The sulfosalicylate, mandelate, and methane-bis(2-hydroxy-3-naphthoate) salts of *p*-aminomethylbenzenesulfonamide (sulfamylon) compare in activity with the hydrochloride of the latter compound in bacteria.

*p*-Aminomethylbenzenesulfonamide and several of its salts are definitely inhibitory to viridans streptococci *in vitro*. The compounds, furthermore, give a suggestion of an antibacterial action against *Streptococcus faecalis*. Under corresponding experimental conditions, sodium sulfathiazole and sodium sulfadiazine are entirely ineffective against these bacteria.

The antibacterial actions of *p*-aminomethylbenzenesulfonamide is not antagonized by *p*-aminobenzoic acid nor by *p*-aminomethylbenzoic acid. The presence of relatively large amounts of vitamins, amino acids, peptones, and carbohydrates in the test medium also fail to interfere with the action of the sulfonamide upon *Clostridium welchii*.

**PUBLIC HEALTH IN MEXICO.** *James E. Perkins*, Division of Communicable Diseases, State Department of Health, Albany.

### EASTERN PENNSYLVANIA BRANCH

ONE HUNDRED AND SEVENTY-FIFTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, PHILADELPHIA, PA., OCTOBER 24, 1944

**THE EFFECT OF TUBERCULOUS AND SENSITIZED SERA AND SERUM FRACTIONS ON THE DEVELOPMENT OF TUBERCLES ON THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICK EMBRYO.** *E. W. Emmart*, National Institute of Health, Bethesda, Md., and *Florence B. Seibert*, Henry Phipps Institute, Philadelphia, Pa.

It has been shown by precipitation tests and by electrophoretic analyses that antibodies were present in the gamma globulin fraction of sera of rabbits sensitized with tuberculin proteins. Electrophoretic analyses of the sera and serum fractions of both experimental and control normal animals were made to determine the relative amounts of the respective globulin frac-

tions. Besides the experiments with whole sera of tuberculous, sensitized, and normal animals, the gamma globulin fraction was isolated from pooled sera of rabbits sensitized with PPD-2 and similarly used *in vitro* and *in vivo*. The tuberculostatic properties were checked *in vivo* by implanting the chorio-allantoic membrane of the 8-day-old chick embryo with suspensions of the bacilli of known concentration mixed with the whole sera or serum fractions. The number of membranes with tubercles and the average number of tubercles per membrane were noted, and the degree of development of the tubercle was studied in microscopic sections of these membranes. It was found that both the sera of rabbits sensitized with

purified tuberculin protein and sera from tuberculous rabbits possessed tuberculo-static properties. The gamma globulin fraction isolated from pooled sera from rabbits sensitized with PPD-2 also retarded the development of tubercles in the chick membranes, whereas the remaining protein fraction without the gamma globulin gave no protection.

**THE ROLE OF THE LYMPHOCYTE IN ANTIBODY FORMATION.** *T. N. Harris and W. E. Ehrlich*, Children's Hospital of Philadelphia, The Philadelphia General Hospital and the Departments of Pediatrics and Pathology, University of Pennsylvania, Philadelphia, Pa.

Following the injection of an antigen into the foot of a rabbit, there appeared a sharp rise in the size of the regional lymph node, and in the output of lymphocytes into the efferent lymph from that node. At the same

time antibodies were found in the efferent lymph.

On separating the lymphocytes from the lymph supernatant it was found that the antibody titer of the contents was substantially higher than that of the surrounding fluid. This difference was greatest at the time of greatest rate of increase of antibody titer in whole lymph.

Incubation of lymphocytes containing one species of antibodies with lymph supernatant containing another species showed that antibodies pass from the cells to the supernatant, to reach approximate equilibrium, not from supernatant to cells. To test the latter possibility under physiologic conditions, normal lymphocytes were allowed to incubate *in vivo* in their own lymph to which antibodies had been added. It was again found that no absorption of antibodies by cells took place.

ONE HUNDRED AND SEVENTY-SIXTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, PHILADELPHIA, PA., NOVEMBER 28, 1944

**THE DEVELOPMENT OF PENICILLIN PRODUCTION.** *G. Raymond Retlew*, Wyeth Inc., West Chester, Pa.

A few of the less known details of the discovery of penicillin by Alexander Fleming were discussed. These were simple observations out of which has developed one of the greatest discoveries to alleviate human suffering. Many of us have seen the phenomena that Fleming observed, but we did not have the vision or imagination to consider its importance. It is this vision and imagination that has made Fleming a great man.

It was pointed out that the excellent observations of Fleming would have been useless if it had not been for a very few men who realized the importance of this work and experimented further with penicillin. The most important work in this respect was carried out by H. W. Florey and the Oxford University group. The development of penicillin would have been retarded for a long time if it had not been fostered by government agencies in the United States. This made possible rapid development of commercial production. Development was ac-

complished in a year that under normal conditions would have taken many years.

**SOME STUDIES ON INFECTIOUS HEPATITIS.**

*John R. Neefe*, Capt., A.U.S., Commission on Measles and Mumps, Army Epidemiological Boards, University of Pennsylvania, Philadelphia, Pa.

Infectious (epidemic) hepatitis and homologous serum hepatitis are among the most important medical problems of the present war. In infectious hepatitis, jaundice occurs 20 to 40 days after exposure, elevations of temperature above 100 F are common, and the disease occurs in epidemic form. In serum hepatitis, jaundice usually occurs 60 to 120 days after the injection of a homologous blood product, fever greater than 100 F is uncommon, and the disease is rarely transmitted to contacts. Indirect evidence indicates that both types of hepatitis are due to a "viruslike" agent. Evidence suggesting that the causative agents are the same and that the observed differences are due to a difference in the route of entry of the agent was presented.

On the other hand, studies on immunity suggest that more than one agent may be concerned in the syndrome of serum hepatitis. The available evidence indicates that both infectious and serum hepatitis produce a homologous immunity of some degree.

Yet an attack of one type does not appear to protect against the other. If confirmed, this suggests that, although the agent of infectious hepatitis may be responsible for some cases of serum hepatitis, one or more other agents also may be concerned.

### MICHIGAN BRANCH

DETROIT, MICHIGAN, NOVEMBER 17, 1944

TWO DISTINCT DIPHTHEROIDS ISOLATED FROM CASES OF INFECTIOUS BOVINE PYELONEPHRITIS. *E. S. Feenstra, F. Thorp, Jr., and C. F. Clark*, Animal Pathology Section, Michigan Agricultural Experiment Station, East Lansing, Michigan.

Some of the morphological, staining, cultural, biological, fermentation, and agglutination properties of nineteen diphtheroids from cases of infectious bovine pyelonephritis have been determined. The tabulated data were studied in an effort to classify these organisms, especially regarding their relationship to *Corynebacterium renale* which has been described as the etiological agent of infectious bovine pyelonephritis. Thirteen of the organisms were indistinguishable from *C. renale* as described by Bergey. The properties of the other six organisms would not seem to allow them to be classified with any of the corynebacteria now described.

The salient differences were that these six cultures required a richer medium for growth, had more curved pairs in stained preparations, digested litmus milk more vigorously, and produced acid in xylose as well as in fructose and glucose.

EFFECT OF A BOOSTER DOSE OF PERTUSSIS VACCINE ON OPSONIC RESPONSE IN CHILDREN. *P. L. Kendrick, G. Eldering, and*

*M. Thompson*, Michigan Department Health Laboratories, Western Michigan Division, Grand Rapids, Michigan.

In order to study the effect of a secondary stimulus or "booster" injection of pertussis vaccine, children who had been included in earlier study groups were given one injection of 0.5 ml of *Hemophilus pertussis* vaccine (10 billion per ml) at the time of their entry into kindergarten. Opsonocytophagic tests were carried out on 187 children before the "booster" injection and again about two months later. Previous tests suggested that a moderately high level of opsonic activity was maintained for as long as 4 or 5 years after primary immunization, regardless of whether plain or alum-precipitated pertussis vaccine, or alum-precipitated combined diphtheria toxoid and pertussis vaccine, had been used. Reactions after the booster infection were on the average 1.6 times as strong as before, and the average increased from the moderate to the strong range. For example, 23 per cent had reactions in the strong range at the time of the first test as compared to 80 per cent after the "booster." The results lend experimental support to the policy of giving a stimulating injection of pertussis vaccine to children just before they start to school, when the chance of exposure to whooping cough is greatly increased.

### EASTERN MISSOURI BRANCH

OSCAR JOHNSON INSTITUTE, SAINT LOUIS, MISSOURI, NOVEMBER 27, 1944

PATENTS RELATING TO PLANTS AND MICROBIOLOGY. *G. W. Freiberg*, Anheuser-Busch, Inc., St. Louis, Mo.

ARE GENE MUTATIONS RESPONSIBLE FOR THE GROWTH FACTOR REQUIREMENTS OF MICROORGANISMS? *E. L. Tatum*, Stanford University, California.

It is proposed that specific genes are responsible, through their primary determination of the specificity of enzymes, for the syntheses of growth factors as well as for all other biochemical reactions in a cell, and that the mutation of certain genes results in the failure to synthesize particular substances which then become growth factors

for the organism concerned. This hypothesis is supported by the production by x-ray and ultraviolet treatment of gene mutations in the mold *Neurospora* with associated specific growth factor requirements, and by the production by similar means of growth-

factor-requiring strains of *Escherichia coli* and *Acetobacter melanogenum*. The operation of mutation and selection provides a reasonable basis for the changes in synthetic capacities of microorganisms which result in their growth factor requirements.

### NEW YORK CITY BRANCH

CORNELL UNIVERSITY MEDICAL COLLEGE, NEW YORK, N. Y., NOVEMBER 14, 1944

**NEW DEVELOPMENTS IN THE APPLICATION OF THE GERMICIDAL LAMP IN INDUSTRIAL BACTERIOLOGY.** *L. J. Buttolph*, General Electric Company, Cleveland, Ohio.

Low-pressure mercury arcs in recently developed glass tubes transmitting about 65 per cent of the bactericidal ultraviolet of wavelength 2537A provide an over-all efficiency about five times that of quartz lamps used in the past for the disinfection of water.

The equal and reciprocal importance of time and intensity in bactericidal exposures was emphasized. An ultraviolet intensity of about 0.01 watt per square foot of surface, or cubic foot of air volume, was suggested as productive of practically complete killing of exposed bacteria in 8 to 10 minutes under adverse practical conditions. Practically complete killing in one minute would be produced by 0.1 watt per square or cubic foot. It was suggested that sporeforming bacteria, yeast cells, and some fungi may require 3 to 10 times this exposure for the same kill, and resistant fungi 50 to 100 times this exposure.

Of the many applications of germicidal ultraviolet to industrial bacteriology, the disinfection of air economically, in great quantity, and to any desired degree, with no effect on the air itself, is unique.

**THE TESTING OF ANAEROBIC TOXINS AND ANTITOXINS BY THE FLOCCULATIVE REACTION.** *Carr H. Parsons*, Lederle Laboratories, Inc., Pearl River, N. Y.

The tetanal flocculative test is now employed for titrating serums, toxins, and toxoids with minimum difficulty with non-specific zones. Careful selection of standard serum and toxin has been found to be of great importance, as well as the use of blending in cases where multiple zones are encountered. The flocculative test has also been found to be specific and practical for serums and toxins of perfringens, septi-

cum, and histolyticum. Recent developments in the production of high potency toxins have made such testing possible and eliminated the necessity for concentrating the antigens. Limited work on sordelli toxin and antitoxin suggests that the flocculative test may be satisfactory. The flocculative test may now be considered an important and dependable test when properly applied for the testing of anaerobic toxins, toxoids, and antitoxins.

**INCIDENCE AND IDENTIFICATION OF PATHOGENIC SHIGELLAE.** *A. J. Weil*, Lederle Laboratories, Inc., Pearl River, N. Y.

Cultural differentiation within the genus remains an essential requirement. The trimethylamine oxide method of Wood is of great importance for speedy differentiation between the Flexner bacillus on the one hand and the Sonne bacillus and the alkalescens variety on the other.

Serological differentiation of species and types of *Shigella* is possible provided that properly absorbed sera are used. Strains occur that are agglutinable only after boiling. That is particularly true for *Shigella ambigua* (Schmitz) and *Shigella alkalescens* and also for some types of the Flexner bacillus, type VI (Newcastle-Boyd-88). The Sonne bacillus shows a peculiar phase variation, and sera containing antibodies to both phases are necessary for complete coverage of that species. The Flexner species is serologically not homogeneous. Absorbed sera for the predominant (primary) type antigens are necessary for differentiation which can be expediently made by slide agglutination. Fourteen such primary antigens have been established which characterize fourteen types corresponding to the five "races" of Andrewes and Inman plus nine types described by Boyd. One of the latter comprises both culturally typical Flexner strains and strains that form slight amounts



of gas ("Newcastle"). In addition, there are several types that have two primary antigens. They correspond to what Andrews and Inman called "subraces," such as VZ. Three types with dual primary antigens have been found up to the present time.

THE USE OF ALBUMIN AS A NUTRIENT FOR *TREPONEMA PALLIDUM*. *Paul A. Little*, Lederle Laboratories, Pearl River, N. Y.

The refined serum albumin that has received attention as a possible substitute for blood plasma, has been found to be a substi-

tute for animal fluids in media used for the cultivation of *Treponema pallidum*. Albumin (0.4 per cent) replaced 10 per cent of blood serum. Concentrations as low as 0.1 per cent of albumin produced detectable growth. Refined serum albumin was used in serum-free media in studies of requirement for anaerobic conditions, for vitamins, and for factors which accelerated growth.

THE PRODUCTION OF INFLUENZA VIRUS VACCINE. *D. W. McKinstry*, Lederle Laboratories, Inc., Pearl River, N. Y.

### NEW JERSEY BRANCH

THE THEOBALD SMITH SOCIETY, RUTGERS UNIVERSITY, NEW BRUNSWICK, NEW JERSEY,  
NOVEMBER 2, 1944

PROBLEMS IN DIPHTHERIA IMMUNITY. *Alfredo Sordelli*, National Department of Health, Buenos Aires, Argentina, S. A.

MICROBIOLOGICAL METHODS FOR THE DETERMINATION OF AMINO ACIDS. I. ASPARTIC ACID AND SERINE. *J. L. Stokes and Marion Gunness*, Research Laboratories, Merck & Co., Inc., Rahway, N. J.

An accurate and specific microbiological method has been developed for the determination of aspartic acid and serine in purified proteins. It is based upon the quantitative response of *Lactobacillus delbrückii* LD5 to those two amino acids as measured by titration of the lactic acid formed. The method satisfies the usual criteria of reliability in that (a) assay values obtained with different dosage levels of test samples agree closely indicating absence of stimulatory or toxic substances; (b) there is good duplication of assay levels on the same protein in different experiments involving different operators and preparation of fresh hydrolyzates; and (c) aspartic acid and serine added to proteins prior to hydrolysis are recovered quantitatively. The microbiological values for the aspartic acid and serine content of casein,  $\beta$ -lactoglobulin, egg albumin, and silk fibroin are in good agreement with those arrived at by recent, improved chemical methods.

A NEW MEMBER OF THE GENUS *ALCALIGENES* AND ITS SYMBIOSIS WITH ACID-FORMING ORGANISMS. *J. A. Anderson*, Rutgers University, New Brunswick, N. J.

A member of the genus *Alcaligenes* has been studied which resembles *Alcaligenes fecalis*, but differs culturally and physiologically from it and other members of this genus. The outstanding characteristic of this organism is its pronounced aciduric quality. This organism, which occurred as a contaminant in a commercial culture of *Lactobacillus bulgaricus*, is a small gram-negative rod which produces a sparse growth in the commoner culture mediums as compared with *A. fecalis*. Colonies on tomato juice or brain heart agar are circular, thin, entire, smooth, moist, grayish, finely granular, 1.5 to 2 mm in diameter. Growth on nutrient agar is poor. Growth in broth is sparse and settles to form a granular precipitate, the supernatant liquid becoming clear. Gelatin is not liquefied. Litmus milk is rendered alkaline, without coagulation, and very slowly peptonized. No growth occurs on potato slants. The organism resembles *A. fecalis* in that it does not utilize carbohydrates or citrates, reduce nitrates, form indole, alter blood, is weakly lipolytic on tributyrin agar, and has an optimum temperature of 37 C. It forms no pigment of any kind.

## INDIANA BRANCH

JORDAN HALL, BUTLER UNIVERSITY, INDIANAPOLIS, INDIANA, NOVEMBER 10, 1944

**MORPHOLOGICAL CHARACTERISTICS OF A PURIFIED THERMOPHILIC CELLULOSE-DECOMPOSING CULTURE.** D. B. Pratt, Biology Department, Purdue University.

The fermentation of cellulose at 65 C as it is usually carried out in tubes can be divided into two stages, a preliminary period during which abundant surface growth is observed but neither pigment nor gas is apparent, and a secondary period in which pigment and gas are formed. Large rods of uniform diameter develop in the upper portion of the liquid during the preliminary stages of the fermentation. These rods are gram-variable when stained according to the Hucker modification of the gram stain. The rods are actively motile at the end of 24 hours. The lower portions of the tube show slower development of a filamentous form. These are gram-negative. With the evolution of gas the forms become mixed and the actively fermenting culture has a heterogeneous nature. As the fermentation proceeds, all rods, both large and filamentous, are gram-negative, and filamentous forms bearing a large, gram-variable, sporelike structure in a completely terminal position become numerous.

In a smear made from an actively fermenting culture a mixture of filamentous rods, large rods, and a few of the forms bearing the large sporelike structures are observed.

**THE NATURE OF "NIBBLED" COLONIES OF BACTERIA RESISTANT TO BACTERIAL VIRUSES.** S. E. Luria, Indiana University, Bloomington, Indiana.

After the action of a bacterial virus (bacteriophage) on a sensitive host, among the secondary colonies of resistant bacteria there often appear "nibbled" colonies. They are distinguished by irregular shape and texture, and are often reduced to thin, barely visible residues. Bacteria isolated from such colonies usually prove as resistant to the virus as the bacteria from regular secondary colonies.

We discovered the occurrence of virus mutants capable of attacking bacteria re-

sistant to the normal viruses. It seemed possible that the nibbled colonies might result from the lytic action of some mutant virus particles on secondary colonies resistant to the normal virus. If so, nibbled colonies should also be obtainable by plating a few cells of a pure resistant bacterial strain with a virus mutant active upon it. By plating together various amounts of bacteria and of virus, it was possible to duplicate all types of nibbled colonies (slightly nibbled, largely nibbled, thin). Whenever nibbled colonies had been found in the secondary growth, it was possible to isolate from the virus at least one mutant active on the bacteria of the secondary growth. Several new mutant viruses were thus isolated from different coli-virus strains.

**INTERFERENCE BETWEEN PARTICLES OF CLOSELY RELATED BACTERIAL VIRUSES.** S. E. Luria, Indiana University, Bloomington, Indiana.

Interference between different viruses has been described for many viruses, including bacteriophages.

For bacterial viruses, occurrence of self-interference has been inferred indirectly. Direct proof is difficult to obtain, because particles of the same virus are indistinguishable and one cannot follow the growth of a certain virus particle in a culture. Isolation of mutant bacterial viruses, indistinguishable from the normal viruses when acting on a common host but active on a new host resistant to the normal viruses, permitted a further study of self-interference. The mutant virus is traceable through its activity on the new host.

Cells of *Escherichia coli*, strain B, susceptible to virus  $\gamma$  and virus  $\gamma'$  (mutant), were infected with virus  $\gamma$ , and immediately after with virus  $\gamma'$ . Then, before lysis took place, the infected bacteria were plated with strain B $\gamma$ , sensitive to virus  $\gamma'$  only. Those bacteria that liberate  $\gamma'$ -particles should produce plaques. It was found that almost all the bacteria infected first with virus  $\gamma$ , then

with virus  $\gamma'$ , failed to liberate any virus  $\gamma'$ . Since we know that viruses  $\gamma$  and  $\gamma'$  are indistinguishable in their action on strain B, these experiments indicate the occurrence of interference between virus particles acting in the same way on the same host cell.

**ANTIBACTERIAL SUBSTANCES FROM PLANTS COLLECTED IN INDIANA.** *Dorothy Sanders, Paul W. Weatherwax, and L. S. McClung*, Indiana University, Botanical and Bacteriological Laboratories, Bloomington, Indiana.

The juice of the plants, or particular portions of them, obtained by a Carver press, was tested for inhibitory activity against *Bacillus subtilis* and *Escherichia coli* using the Oxford cup technique. Representatives (1 to 50 species) of the following families have been included: Plantaginaceae, Ranunculaceae, Gramineae, Araceae, Solanaceae, Pahayaceae, Phylolaccaceae, Polygonaceae, Liliaceae, Compositae, Asclepiadaceae, Violaceae, Menispermaceae, Labiatae, Euphorbiaceae, Caprifoliaceae, Saxifragaceae, Oxalidaceae, Iridaceae, Onagraceae, Leguminosae, Umbelliferae, Anacardiaceae, Ulmaceae, Apocynaceae, Rosaceae, Solicaceae, Aceraceae, Celastraceae, Alismaceae, Anonaceae, Magnoliaceae, Nymphaeaceae, Juglandaceae, Tiliaceae, Vitaceae, Ericaceae, Polypodiaceae, Osmundaceae, Acanthaceae, Celastraceae, Convolvulaceae, Primulaceae, Urticaceae, Typhaceae, Scrophylariaceae, Balsaminaceae, Simarubiaceae, Lauraceae, Bignoniaceae, Rubiaceae, and Martyniaceae.

Although about 15 of approximately 150 specimens tested show some degree of inhibitory activity against one or both test organisms, no sample has been encountered which gave exceptionally high values. Perhaps the greatest activity was shown by extracts of the common ragweed, *Ambrosia elatior*, though this was not true of the giant ragweed, *Ambrosia trifida*. It should be mentioned that in many instances a very marked stimulation of growth of the test organisms was evident.

**ON THE PREPARATION OF INFLUENZA VIRUS VACCINE.** *H. M. Powell*, Lilly Research Laboratories, Indianapolis 6, Indiana.

We have prepared types A and B influenza

virus in the usual way on ten-day incubated fertile hen eggs. Such virus, harvested after an incubation period of about 48 hours, has exhibited satisfactory hemagglutinating potency, generally a 1:256 dilution giving a strong reaction against chicken red blood cells. Also, virulence for young Swiss mice was satisfactory, the titers for type A and type B being about 1:100,000 and 1:10,000, respectively.

Virus has been used in the nonconcentrated state, and as tenfold concentrated material, as vaccine. Since the nonconcentrated material was experimented with first, its immunizing action as modified by different inactivating and preservative agents was determined. Swiss mice were treated with two doses of vaccine intraperitoneally with an interval spanning a week and then, one week after the last dose of vaccine, dilutions of active virus were administered intranasally to immunized and control mice. All vaccines were tested after being heated for one week and one month, respectively, at 37 C.

All vaccines containing "merthiolate" (sodium ethyl mercuri thiosalicylate, Lilly) or formalin, alone or in combination, immunized well. All vaccines containing phenol or saturated sodium chloride had weak immunizing action.

**A STUDY OF NONTOXIC STRAINS OF CLOSTRIDIUM TETANI.** *Ruth Toabe and L. S. McClung*, Indiana University, Bacteriological Laboratories, Bloomington, Indiana.

At the request of Dr. J. H. Mueller, from whom the culture was received, a study has been made of a strain of *Clostridium tetani* which lost the property of toxin production following a period of serial daily transfers in meat infusion glucose broth. In addition, 10 other nontoxic and 33 toxic strains, mostly received from Janet Gunnison, have been included in our series. The 10 strains are presumed to have been nontoxic on original isolation but possess other characters, including agglutinating antigens, which are normal for the species. All strains have been studied with respect to the following: cell morphology, spore formation, motility, physiological reactions, and colony type.

To date, no significant difference has been

observed with respect to those characters in the various strains, except that in the serial passage culture spore formation is markedly retarded. This is not true of the original culture from which this strain was derived. Rough-smooth dissociation apparently has not occurred. An attempt to repeat the phenomenon of loss of toxicity by serial passage in thioglycollate broth and other studies on this and additional strains are in progress.

LABORATORY DIAGNOSIS OF RICKETTSIAL DISEASES. *George D. Brigham.*

The various techniques for laboratory aids in establishing a diagnosis of Rickettsial infection were reviewed. The relative importance of each procedure in such laboratory work was evaluated. Suggested helpful points for given cases were emphasized.

PATHOGENESIS, DIAGNOSIS, AND TREATMENT OF *CLOSTRIDIUM WELCHII* INFECTION.

*Lyle A. Weed*, Department of Bacteriology and Department of Research, Indiana University Medical Center, Indianapolis, Indiana.

The conditions commonly encountered in developing gas gangrene were briefly reviewed. The reasons were presented for believing most cases are mixed infections. The possible roles of the associated organisms were discussed. Original data were presented showing lack of symbiotic or synergistic effects of some common aerobes when mixed with *Clostridium welchii*. The role of soluble toxin was discussed and data presented to show the great irregularities of antitoxin therapy when the disease is moderately well developed. Other forms of therapy were briefly reviewed. Attention was called to the lack of satisfactory recognized therapeutic procedures and the need of further work to understand the fundamental physiological reactions related to death resulting from infections due to *C. welchii*.



# THE MORPHOLOGY OF BACTERIUM TULARENSE

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Divergent views exist concerning the morphology of *Bacterium tularense*. McCoy and Chapin's (1912) original description depicted an apparently immotile, small, questionably encapsulated, pleomorphic organism occasionally presenting enlarged, irregular, and apparent involution forms; sometimes with predominant large globular forms. Ohara, Kobayashi, and Kudo (1935) claimed to have demonstrated flagella on both Japanese and American strains, stated that motility was observed repeatedly, and described clubbed, comma-shaped, dumbbell, and triangular forms. They further stated, "The more virulent is the bacterium, the greater is the pleomorphism"; also, "Virulence, pleomorphism, and motion are closely related and vary together." Galli-Valerio (1938), after working with cultures isolated by Drbohlav in Czechoslovakia, stated that both coccoid and bacillary forms were absolutely immotile and that no flagella were demonstrable by the Casares-Gil stain. He also failed to find any enlarged, elongated, filamentous, or involution forms. Most European language reports and textbooks omit all reference to flagella, state that the bacterium is non-motile, present inadequate descriptions of the extraordinary pleomorphism, give scant mention to encapsulation except as an occasional finding in tissues or in tissue smears, and classify the organism among either *Pasturella* or *Brucella*.

Since the most extensive studies on morphology were conducted by Ohara and his associates, we reviewed thoroughly the Japanese literature. Ota (1936) states that Kudo and Kobayashi, working in Ohara's laboratory, first demonstrated a single polar flagellum in 1934, using the silver deposition method of Nishigawa and Sugahara (apparently the same as the Saisawa-Sugawara method mentioned by Ohara). They also observed capsules. Ota confirmed this work. He demonstrated flagella also with Victoria blue (4R), Burri's India ink method, and by his own modification of Benian's Congo red method. In his experience the methods of Loeffler, Benian, Zettnow, Inouye, Yokota, and Uyenno either failed entirely or showed few poorly stained flagella. Under dark-field illumination, "Refractile flagella were demonstrated." With regard to motility, "I found some actively motile, definitely changing their position." Capsules were well demonstrated by Ota's modification of Benian's method, mercurochrome negative staining, and by Gin's India-ink carbol-thionine method. The methods of Johnne, Wadsworth, Hiss, Welch, and Friedlander were said to stain them poorly or not at all. Ota stained *Bacterium tularense* and Yato-byo bacteria, also their flagella, in tissues with the Levaditi method. Successful preparations were made from human lymph node and skin, guinea pig spleen, and rabbit liver.

<sup>1</sup> In partial fulfillment of the thesis requirements for the degree of Doctor of Philosophy.

<sup>2</sup> Captain, S. C., A. U. S.

Ohara, Kobayashi, and Kudo used the Saisawa-Sugawara silver deposition method. They described the typical *Bacterium tularensis* as a coccus or bacillus with a single polar flagellum. Yato-byo bacteria were said to have longer flagella than American strains of *Bacterium tularensis*. They noted bacterial forms connected by "flagella," also apparent multiple flagella, and instances of three cocci united by "flagella," but assumed that these images were preparational artifacts. Their American strains were 38, Hen, Va, and Col, all received originally from E. Francis, and sent to Kudo by us.

With regard to motility these workers stated that the bacterium "has a certain active movement," and that the degree of motility was proportionally related to virulence. They described its motility as "slower than the movement of cholera vibrio and much quicker than that of typhoid bacillus." Addition of a drop of mercuric chloride solution to the bacterial suspension was said to cause motility to disappear slowly, leaving only Brownian movement.

Ohara (1940) further mentioned the "specific active movement" and stated that the organism is monotrichate with a polar flagellum, also a capsule. A privately printed reprint of this complete paper, containing excellent photomicrographs, was presented to members of the Third International Congress of Microbiology. Ohara's statement that the marked difference in severity between tularemia and yato-byo is not due to inherent differences in virulence between Japanese and American strains, but rather due to the constitution of the Japanese people, should be modified in accordance with the experience related by Ota, who describes in detail the clinical course of disease in his laboratory technician infected by the American strain, Hen. The patient developed pleurisy and pneumonia with bloody sputum during the first week. During the second week he was irrational, with fever from 40.4 to 41.9 C (107.4 F), and had anuria for 7 days. Seven thoracenteses were performed. He was febrile for 64 days and disabled for more than 3 months. Agglutinin titers ascended from zero in the first week to 1:640 in the third week, and 1:2,560 in the sixth week of disease. The infecting strain was recovered from the pleural fluid.

We wish to record results of a systematic study of the morphology of this microorganism. Forty-three strains were examined, 21 from our collection and 22 others supplied by Dr. Edward Francis from the National Institute of Health. The histories of these strains are shown in table 1. Attention is directed to the wide range in geographic origin, to the diversity of pathologic sources, to an almost complete series of annual original isolations from 1920 to 1942, and to the periods of cultivation on artificial media which extended from 22 years to 2 days. The virulence range extended from maximal—killing all mice, guinea pigs, and rabbits within 3 to 5 days after dermal inoculation or after parenteral injection of 0.5 ml of 48- to 72-hour cultures in saline dilution of  $T-500 \times 10^{-2}$ —to absolute nonvirulence—no illness in any of 12 mice and 12 guinea pigs after injection of approximately 12 billions of bacteria from a 48-hour culture into each animal.

#### METHODS OF STUDY

*Motility.* Motility was studied in salt-solution hanging drops and in vaseline-paraffin-luted cover slip preparations, by both direct illumination and dark-field

TABLE 1  
*Histories of the strains examined*

DESIGNATION OF CULTURE	YEAR OF ORIGINAL ISOLATION	PATHOLOGIC SOURCE	GEOGRAPHIC SOURCE	INTERVAL BETWEEN LAST ANIMAL ISOLATION AND OUR EXAMINATIONS
38	1920	Human Ly. node	Utah	22 years
26	1921	Human blood	Utah	21 "
SF	1922	Ground squirrel	Calif.	20 "
T	1923	Wood tick	Montana	19 "
V	1924	Human spleen	D. C.	11 "
Sn	1925	Wild hare	Montana	17 "
Jap	1926	Human Ly. node	Japan	16 "
LR	1927	Human ascitic Fl.	Arkansas	15 "
Max	1928	Human Ly. node	Russia	14 "
Russ	1928	" " "	"	14 "
Can	1930	Wild hare	Canada	12 "
Ohara	1931	Unspecified	Japan	11 "
Ri	1932	Human pus	Virginia	10 "
Fox I	1933	Gray fox spleen	Minnesota	9 "
Col	1933	Human pleural Fl.	So. Car.	8 "
Ll	1934	Human pus	Canada	8 "
Tol	1934	Human blood	Ohio	7 "
T-418	1934	Human blood	Ohio	6 "
L.C.	1935	Human ulcer	Virginia	7 "
H.D.	1935	Human eye	Austria	7 "
Md	1936	Human pleura	Maryland	6 "
PF	1936	Human lung	Ohio	2 "
Chr	1937	Human pus	Ohio	12 days
Sto	1937	Human blood	Ohio	5 years
Chil	1937	Human pus	Ohio	4 "
Trot	1937	Human lung	Ohio	8 months
Die	1938	Human ulcer	Calif.	2 years
Memp	1938	Human Ly. node	Tenn.	10 days
De P	1938	" " "	Ohio	2 years
Pi	1938	Human blood	Ohio	8 days
Pack	1939	Human sputum	D. C.	2 years
V.F.	1940	Human pleural Fl.	Tenn.	2 "
Hugh	1940	Human ulcer	Ohio	2 "
Clem	1940	Human blood	Ohio	2 "
Fox	1940	" "	Ohio	1 "
Broo	1941	Human spleen	New York	1 "
Fish	1941	Human ulcer	Ohio	10 months
Well	1941	Human pus	Ohio	9 "
Fran	1941	Human sputum	Ohio	5 "
Gib	1941	Human ulcer	Ohio	3 "
Schu	1941	" "	Ohio	3 days
Chur	1941	Human lung	Ohio	2 "
Bish	1942	Human pleural Fl.	Tenn.	4 months

examination. Our associates, Tamura and Gibby (1943), provided semisynthetic and synthetic liquid media in which heavy growth develops from inocula of approximately 15 organisms to 5 ml of media. Observations on morphology and motility were also made of cultures in these media. Some strains had been



propagated in liquid media for months; others were sown into liquid and observation preparations were made daily for 8 days, the average period of multiplication.

*Fixed and stained smears.* The methods used to stain for flagella were those of Gray, Fontana-Tribondeau, Saisawa-Sugawara, Casares-Gil, Von Ermengem, Leifson, Inouye, and Weiss. For capsule staining we used the methods of Churchman, Welch, Anthony, Wherry, and Hiss.

*Preparation of bacterial suspensions.* Most cultures were grown on solid media for 24 to 72 hours. Physiologic salt solution proved better than distilled water as a suspending medium. Satisfactory suspensions were made in either of two ways:

1. A small amount of surface growth was removed with a loop and very gently shaken into a tube of salt solution. The tube was incubated at 37 C for 1 to 2 hours until a uniform suspension resulted. Occasional gentle rotation accelerated this process, but shaking broke off most "flagella." The cohesive nature of the growth made it difficult to dislodge it from the loop.

2. One or 2 ml of salt solution were pipetted into the end of a culture slant, the tube was incubated until there was visible turbidity, then some of this suspension was pipetted to another tube of salt solution. This was the better method.

These suspensions were good for "flagella" demonstration for at least 3 days, but "capsules" were best shown during the first 6 hours. Preparations were also made from growth in liquid media.

Thin air-dried films were stained on both slides and cover slips. Preheating slides in a gas flame gave preparations with the clearest backgrounds. Although the Gray stain must be mixed for each day, we found it left less background deposit if it was allowed to stand 1 hour before using. Beyond this, the technique was simply that always necessary for success with special staining methods—scrupulously clean glassware, experience with the methods, and patience.

*Supravital staining of living organisms.* Each strain was also studied by supravital staining, either suspended in saline from growth on solid medium or directly in gelatin-hydrolyzate liquid medium. More than 30 dyes were used, but only Nile blue sulphate, Hofmann's violet (Dr. G. Grüber & Co.), Janus green B, malachite green, safranin O, gentian violet, and Bismarck brown Y proved useful. These were used chiefly as saturated solutions in saline, adding 1 small loopful of freshly prepared and filtered dye to an equivalent amount of suspension or culture on a slide, mixing, covering with cover slip, and sealing. Malachite green was used in 2, 3, and 5 per cent aqueous solutions. Examinations were made both by direct illumination and by dark field. Victoria blue (4R) also would probably have been satisfactory, judging from Ota's experience with fixed smears. We were unable to obtain it.

Since most of our photomicrographs of living organisms required exposures in excess of 15 seconds, we had difficulty with Brownian movement. Cover slips could not be rolled down close without producing numerous artifacts. Our most

successful method to avoid blurred images was to melt a drop or two of ordinary 14 per cent gelatin medium by gentle heat in the center of the slides, heating until the gelatin flowed into a thin, even layer. After solidification of the gelatin, preparations were made and sealed as usual. Organisms entrapped in the lower viscous layer were motionless and not deformed.

*Examination of blood from infected rodents.* Heart blood drawn from moribund or dead experimentally infected mice and guinea pigs was examined by direct illumination and by dark field in sealed cover slips immediately as drawn, diluted in sterile distilled water, in sterile saline, and in gelatin hydrolyzate medium; all both unstained and supravitality stained.

*Control of methods of study by application to other bacteria.* Since formation of artifacts by technical procedures is an ever-present hazard in studies on morphology, especially when new or infrequently used methods are employed, we subjected one or more strains of other bacteria to the special staining methods listed above. Strains of *Escherichia coli*, *Eberthella typhosa*, *Pseudomonas aeruginosa*, *Brucella melitensis*, *Pasteurella pestis*, and *Pasteurella pseudotuberculosis-rodentium* failed to reveal any new or unusual morphologic features when examined by these methods.

#### RESULTS OF OBSERVATIONS OF STAINED SMEARS

Examination of fixed smears stained by customary dyes, or even by special stains, is at best a poor method to reveal the morphology of this organism. No other method used produced as many deceptive images or as many artifacts except the examination of heart blood from infected rodents. Nevertheless the following observations are recorded, and illustrated in plates I and II, to provide comparison and correlation between previous studies and the results of more suitable methods of examination that follow.

*Demonstration of structures that simulated capsules.* Structures that simulated capsules were best demonstrated by either the Gray or the Saisawa-Sugawara flagella stains, less well by the Fontana-Tribondeau and Casares-Gil stains. We failed to demonstrate them with the Churchman, Welch, Wherry, Anthony, or Hiss capsule stains, though modifications of each were tried.

These structures were demonstrated on every strain. Although they looked like capsules to casual inspection, critical observation showed that they were not extracellular structures but actually cell walls, a feature better demonstrated by other methods of study that follow. Here it will suffice to note that the coccoid form of the organism, whether of small, medium, or large size, usually showed the cell wall. It was rarely seen on bacillary forms. When coccoid and bacillary forms appeared in chain formation it was usual to find cell walls only on the former. The very large coccoid forms often showed thick cell walls, and frequently two or three minute, circular, clear areas, or, sometimes, indentations were noted within such cell walls, usually almost exactly 120° of arc apart. Whenever such forms possessed "flagella," these structures seemed to arise from these circular areas.

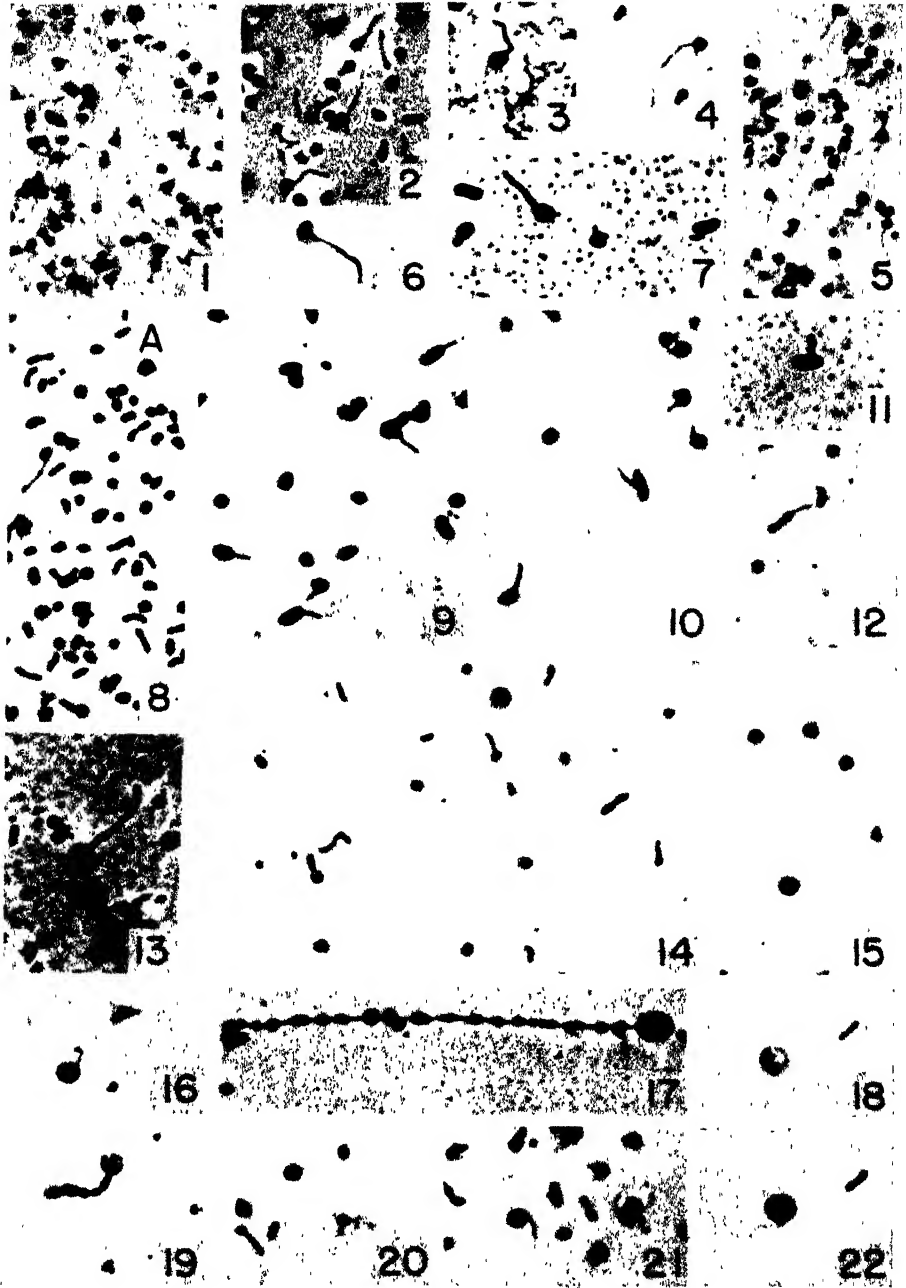


PLATE I

Photomicrographs of stained smears. Reproduced with slight reduction from initial magnifications of 2,250 diameters.

1. Chri strain, virulent. Saisawa-Sugawara silver stain. Usual coccoid forms. Note how closely the filaments resemble flagella; also the small budding form near top center. These coccoid forms average  $0.45 \mu$  in diameter.

Cell walls were formed by both avirulent and virulent strains; by recently isolated cultures as well as by old cultures propagated only on media for more than 20 years.

*Involution forms.* Classical involution forms were observed in stained smears in about half of the strains. Although typical examples were seen and photographed in an avirulent strain propagated only on media for 22 years, they occurred more frequently in virulent strains, and most frequently in recently isolated cultures. Dumbbell, bean-shaped, spermatozoonlike, and many bizarre forms were seen, as well as L-shaped and irregularly knobby globoid forms. Long filamentous forms like those produced by the plague bacillus were not observed, but many shorter and bizarre filamented forms were seen, some of which are reproduced. It can be said here that none of these apparently typical forms were true involution forms, and that their real nature will be demonstrated in succeeding paragraphs.

*The appearance of flagella in stained preparations.* Very fine filamentous

- 2 Col strain, virulent. S-S stain. Slide prepared by Kobayashi, in Fukushima, in 1935; our photomicrograph. Note budding, which the Japanese overlooked, also the minimal reproductive units on filaments near lower left center.
3. 38 strain, avirulent. Gray stain. No animal passage since 1920.
- 4 Schu strain, virulent. Gray stain. Note two M.R.U. near middle of filament. Note minute second filament, also budding of the lower coccoid form.
5. H D strain, virulent. Casares-Gil stain. Note similarity of this Austrian strain to Ohio strain of figure 1. Filamented coccoid forms.
6. 38 strain, avirulent. S-S stain. Note filament extending through cell wall.
7. Schu strain. Gray stain. Short and long filaments. Budding at left. Heavily mordanted preparation.
8. Same. Long filament connects usual coccoid form with a M.R.U. Cell walls on many "cocci." Drumstick form at bottom. At "A" is shown a heavily stained cell wall. Note evidence of budding, also suggestion of division by fission.
- 9, 10, 11 Same. Filamented coccoid and bacillary forms. Attachments of filaments in various locations.
12. Schu strain. Casares-Gil stain. Bacillary form with terminal filament.
13. Schu strain. Gray stain, heavily mordanted. Thick filament on coccoid form.
- 14 Same. Lightly mordanted. Delicate cell walls on large and small "cocci." Drumstick forms.
- 15 Same. False "flagella" produced by protoplasmic streamers from ruptured cells. At bottom, part of a filament is seen within the cell wall, opposite the site of rupture.
16. Same. Globule showing cell wall and a filament with a M.R.U. at tip. Coccoid form is about  $1\ \mu$  in diameter.
17. Max strain. Casares-Gil stain. Long beaded chain with filamentous linkage, probably formed by budding from the globus.
18. Schu strain. Gray stain. Focused to show a segmented globus containing 2 coccoid forms. The filament of the upper "coccus" is within the cell wall and shows a bulbous tip.
19. Same. Sessile budding producing nonfilamented chains. Note that coccoid forms show cell walls and that bacillary forms do not. This is usual.
20. Same. Small globule with well-stained cell wall. Drumstick form at lower left.
21. Same. Traumatized coccoid forms with ruptured cell walls (cf. 15), showing filaments partially uncoiled from original enclosed position.
22. Same globus as in figure 18. Focused deeper to show external protrusion of filament of lower small coccoid form.

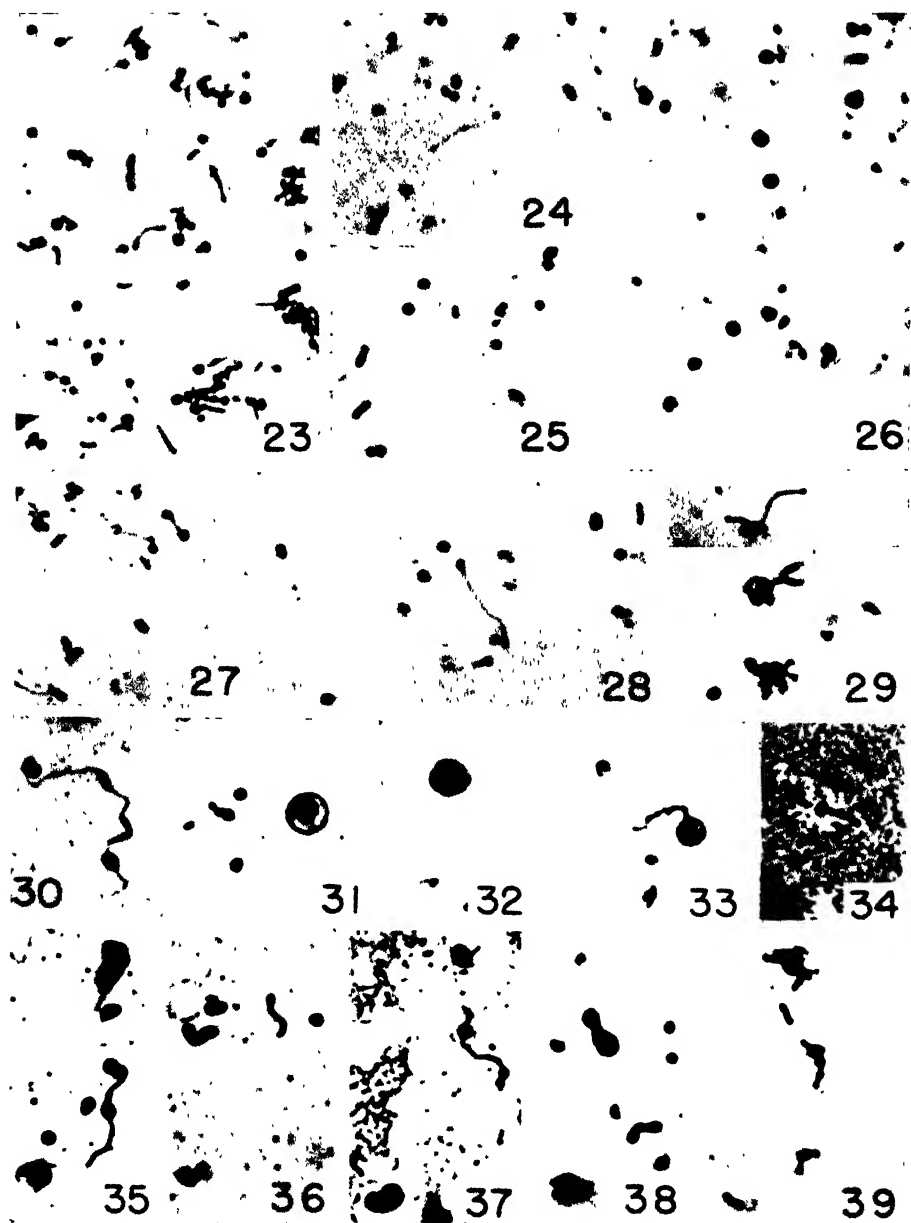


PLATE II

Photomicrographs of stained smears. Reproduced with slight reduction from initial magnification of 2,250 diameters.

23. Die strain. S-S stain. Note frequent filamentous linkage of various forms. A filamented triangular zoogloealike mass at lower left. Many M.R.U. can be seen arising within filaments.
24. Ri strain. S-S stain. Filamentous connection (production?) of M.R.U.
25. Same. Note length of filament.

structures, identical with those the Japanese students call "flagella," were observed on every strain. They were best demonstrated by the Gray, Casares-Gil, and Saisawa-Sugawara staining methods. Excellent preparations were made by each method, and occasional good ones by the Fontana-Tribondeau stain. We were unable to demonstrate these structures with the staining methods of Von Ermengem, Leifson, Inouye, or Weiss. Prolonged staining with Wright's stain, Giemsa stain, or hematoxylin occasionally demonstrated them, but poorly. The pale colors, and the minute size owing to absence of previous mordanting, made such preparations poor subjects for photography. One plate was obtained from which measurements could be made.

In the unmordanted state a coccoid form of the usual size has a diameter of about  $0.45\ \mu$ . The form early noted by us as the "minute coccus" was not seen in unmordanted preparations. After heavy mordanting these forms have a diameter not greater than  $0.2\ \mu$ , and cannot be distinguished from precipitated stain unless they possess a "flagellum." Giant "cocci," and some so-called involution forms, have diameters as large as  $3.0\ \mu$ . Length of "flagellum" varies greatly, from  $0.5\ \mu$  to  $8.0\ \mu$  or longer. A few specimens stained without previous mordanting showed "flagella" of uniform thickness, but really so thin that they were discernible with difficulty under critical illumination. They appeared to be not greater than  $0.05\ \mu$  in thickness.

Although Ohara stated that the longer "flagellum" of Yato-byo bacteria differentiates them from *Bacterium tularense*, we were unable to find significant differences in length among our 43 strains.

These flagellalike structures were seen most frequently on coccoid forms, regardless of size; less frequently on bacillary forms. Many showed bulbous tips

26. 38 strain. S-S stain. Note well-stained cell walls on this old laboratory strain.
27. Ri strain. S-S stain. Filamentous linkage between various forms.
28. Same. Illustrates filamentous development of coccoid and bacillary forms. Note M.R.U. at end of filament, at left.
29. Memp strain. Fontana-Tribondeau stain.  
Upper. Pullulation of filaments containing M.R.U.  
Middle. Globus containing 2 coccoid bodies, the whole attached to a branched mycelium. Strongly suggestive of a sporangiophore, sporangium, and 2 sporangiospores.  
Lower. Radial filamentation at right and below; continuous sessile budding at left upper.
30. Max strain. Casares-Gil stain. Filamentous development of coccoid and bacillary forms, and M.R.U.
31. Schu strain. Gray stain. A  $2.5\ \mu$  globus with thick cell wall. Note eccentric chromatin, and indentation in cell wall that denotes a potential budding site.
32. Same slide. Globus with extremely delicate cell wall.
33. Schu strain. Gray stain. Globule with a chain of small coccoid and bacillary forms produced by sessile budding. Note additional potential budding site on right edge of globule, marked by tiny ring in cell wall.
34. Max strain. Casares-Gil stain, heavily mordanted. Drumstick form.
35. Schu strain. Gray stain. Filamented chain.
36. 38 strain. S-S stain. So-called involution forms, spermatozoon form.
37. Same. Globoid "involution" forms, and filamented chain of coccoid forms.
38. Same. More so-called "involution" forms.
39. Memp strain. Fontana-Tribondeau stain. Sessile and filamentous budding, branching.

at the free end. Others frequently showed another coccoid or bacillary form at this end, giving the appearance of two bacteria united by a fine filament. Other fine filamentous structures united 3, 4, 5, or more than 20 bodies of either coccoid or bacillary shapes. The appearance of multiple flagella was also noted. At first we thought all such images were preparational artifacts and, indeed, by judicious variations in methods of preparation, especially in depth of mordanting and staining, most such images from a given suspension could be resolved into apparently monotrichate bacteria lying very near free "flagella," the free end of another "flagellum," and so forth. Although light mordanting revealed many such images as artifacts, it did not dispose of the matter of various bacterial forms united by fine filaments. Every strain continued to show united forms under critically controlled variations in technique.

Differentiation between flagella-sized filaments and protoplasmic streamers due to traumatic rupture of cell walls was easily made. Filaments stained evenly and deeply, with sides parallel throughout their length. Protoplasmic streamers stained lightly, often unevenly, and tapered from a broad base near the soma to fine single or multiple distal tips.

When the so-called "flagella" appeared on bacteria with cell walls, they sometimes seemed to arise from the soma and penetrate through the cell wall. Others seemed to arise from the cell wall. Similar appearances in relation to the "capsules" of *Bacillus subtilis* were noted and discussed by Churchman (1933). With regard to *Bacterium tularensis*, we believe this disparateness is due to variations in staining technique. From a single suspension we made preparations that showed first one appearance, then the other. We occasionally saw both forms on the same slide, the "flagellum" not visible through the cell wall in lightly stained areas, but well shown within it in more heavily stained areas.

In some coccoid forms of *Bacterium tularensis* the "flagellum" was very clearly seen coiled within the intact cell wall. This is shown in some of the photomicrographs. In the largest forms the internal location of the filament was exceptionally well shown, where they commonly heavily accentuated about one third of the internal perimeter of the cell wall. If cell walls had been broken by trauma, these filamentous structures could frequently be seen partially uncoiled from the original position and easily differentiated from the associated protoplasmic streamers. One photomicrograph shows this arrangement very clearly.

The coccoid form was the one most frequently seen, and most "flagella" and most cell walls were seen on this form. It was the only form in which "flagella" were observed within cell walls. One might argue that, if the filaments observed were true flagella, the almost universal failure of skilled observers to see motility of *Bacterium tularensis* might result in part from the frequent internal location of flagella, especially since more than 3 hours are required for cell walls to disintegrate in salt solution. This hypothesis implies that proper or suitable conditions for observation of motility might never have been secured, an hypothesis which was readily made untenable by our study of living cultures in extremely favorable liquid media by dark-field illumination and by supravital staining.

## OBSERVATIONS ON LIVING CULTURES UNDER DARK-FIELD ILLUMINATION

All forms previously seen in stained preparations were readily recognized under the dark field. The "minute coccoid forms" were seen singly, in groups, in zooglee-like masses, and at either or both ends, or along the courses of extremely delicate nonmotile filaments. They varied in size from just smaller than the usual coccoid forms to such minute bodies that neither size nor shape was discernible. Many control preparations showed that these bodies were not present in our saline solution or in uninoculated liquid media. Coccoid forms of usual  $0.45$  to  $0.5\ \mu$  size were not spherical but rather crescentically obovoid, shaped somewhat like a segment of an orange, with one end larger and rounder than the other. The refractile chromatin body was seldom central; usually peripherally located within a scarcely discernible, delicate cell wall. A moderate number of coccoid forms of this size revealed small budding spherules. Some were sessile; others pedunculated or attached by extremely fine filaments. Coccoid forms of  $1.0$  to  $1.5\ \mu$  in diameter were more spherical, usually contained one or more highly refractile chromatin particles eccentrically located, and frequently exhibited both sessile and filamentous buds. The bipolar forms of the stained preparations were seen to be diploforms of the  $0.5\ \mu$  size, shaped like a figure 8 in the dark field view, with a chromatin particle in each end, or bacillary forms with polar chromatin particles. The "giant cocci" were actually globular forms  $2.0$  and  $3.5\ \mu$  in diameter. They were occasionally twinned, sharing the largest chromatin particle at the junction of the rounded free surfaces with the plane interface surface. In addition, many globi of this size presented moderate to numerous peripherally located, smaller, refractile particles, often arranged in meridional or equatorial belts. It was these globular forms, especially when actively budding, together with certain bacillary-like forms to be noted presently, that appeared in stained smears as the so-called "involution forms."

Bacillary forms were present in many sizes and shapes. In general, they were not strictly bacillary in shape with the short diameters of approximately equal size. They were taeniform, or ribbon-shaped, with one short diameter very much shorter than the other. In addition to the common flattened "bacilli" we noted a less frequent long form which was almost round, or quite round, in transverse diameter. These appeared like true filamentous forms of bacillary dimensions. They differed in being more highly refractile throughout their length, often tapered to a smaller diameter at one end. These also showed frequent budding.

All sizes of "cocci" and "bacilli" appeared singly, in diploform, in short or long chains, and sometimes in chain formation connected by filaments of various lengths. The predominant form was almost always the  $0.45\ \mu$  "coccus." All 43 strains showed the same range of morphologic diversity.

The "flagella" described and photographed by Ohara and his associates were readily seen. Their appearance in dark-field preparations was identical with that in fixed preparations stained by the Japanese, in similar preparations made by us, and in the photomicrographs published by Ohara as well as in our own.



However, all such "flagella" were absolutely nonmotile, whether observed in water, salt solution, or liquid media. We watched many living preparations all day for many days, making a fresh one for a single culture every day throughout its period of multiplication. We saw thousands of these flagella-sized filamentous structures attached to all observed forms of the organism, but they were invariably nonmotile. The bacteria multiplied well in the liquid medium, and there was seldom doubt concerning viability of cells observed. Suspended dead cells, with or without "flagella," were mere shadow outlines of living ones but all, living or dead, showed only Brownian movement. The easiest way to locate a cell with a long "flagellum" was to look for one that, for its diameter, had less Brownian movement than would be expected. The longer the filament the more it restricted Brownian movement in rate and amplitude. If there was also an organism at the other end of the filament it behaved as a drag anchor, permitting almost no Brownian movement. Multiple "flagellated" living forms were seen, confirming our findings in stained preparations.

In living cultures filaments of flagellar size were seldom attached to only one organism. In stained preparations this appearance was very frequent, simulating true flagella, but it seems almost certain that this was an artifact caused by trauma incident to the preparation of dried films. In cultures the great majority of fine filaments connected two or more organisms of any of the forms noted.

We can offer a possible explanation for the "peculiar motion" so frequently mentioned by Ohara and his co-workers. Some bacillary forms, and all refractile rounded filamentous forms, exhibited active flexional movements. These squirmings rarely accomplished any motion of translation, but occasionally an especially vigorous bend effected a change in position of a micron or two. We frequently left such forms located in a field for an hour, to return and find the "bacillus" or "filament" not more than one-half field diameter from the point at which we left it. Ohara writes of thin and thick flagella. From our observations we believe all "thick flagella" are slender bacillary forms or the round refractile filaments. When a long bacillary form or filament had a coccoid form at one end, noted as the "drumstick" forms in our photomicrographs, the flexional movements of the rod-shaped section gave the appearance of a thick flagellum. We watched many such forms, and the greatest rate of movement of translation recorded was of the order of 15  $\mu$  per hour. We confirm the constant presence of flagella-sized filamentous structures, true filaments of bacillary transverse diameter, small, medium, and large globules, and extraordinary pleomorphism, but not motility or encapsulation.

Figure 1 shows drawings from dark-field observations of living cultures of some strains at various stages of growth. It must not be inferred that the forms shown were present in every field. At least one example of every form drawn was observed in every strain, but the filamented and other bizarre forms represented only a small fraction of the total populations. The most numerous forms seen during any of the first 8 days of cultivation were always the coccoid and bacillary forms. Many of these were not filamented.

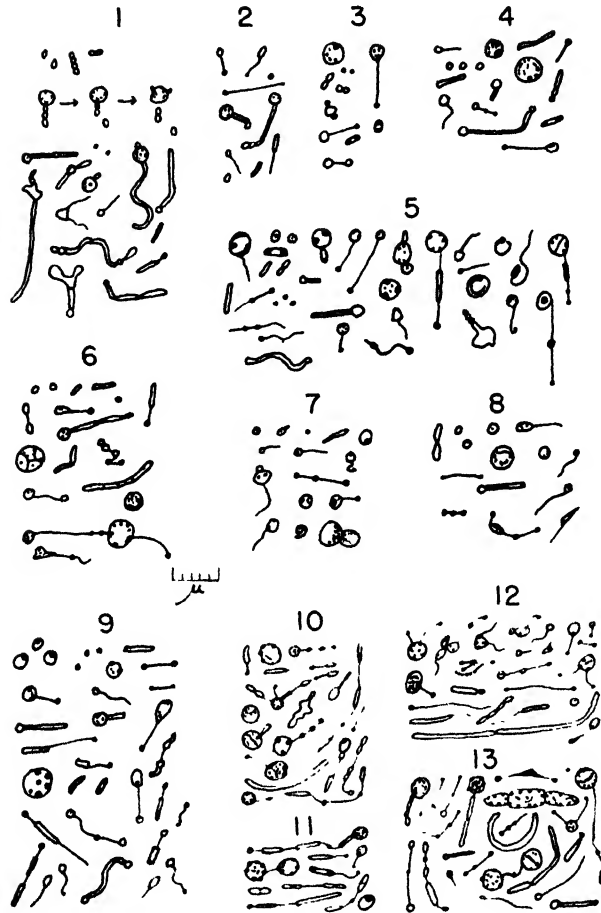


FIG. 1. SELECTED MORPHOLOGIC UNITS OF SEVERAL STRAINS OF BACTERIUM TULARENSE

Drawn from observations of living cultures in gelatin hydrolyzate media under dark-field illumination. The preparation from which no. 9 was drawn was made by suspending in saline solution a small amount of growth removed from a blood cystine agar slant; it is included to show that the same forms are produced by cultivation on the standard solid medium.

1. Strain Memp at 19 hours of incubation. Thirty to 40 minutes elapsed between the successive stages indicated by the arrows. Note reproduction by budding.

2. Strain Memp, 44 hours.

3. Strain Memp, 90 hours.

4. Strain Memp, 5 days.

5. Strain Memp, 6 days. Note filament coiled within cell wall of one globule.

6. Strain Jap, 25 hours. Note filamented zooglee-like mass at bottom.

7. Strain Ohara, 48 hours.

8. Strain Ohara, 72 hours. At bottom note filaments derived from an ill-defined zooglee-like mass.

9. Strain Schu in saline solution; 72-hour culture. Signet ring forms, at upper left, were very common in all strains.

10. Strain Paek, 48 hours. Note evidence of reproduction by pullulation of filaments.

11. Strain Paek, 72 hours. Some forms shown near bottom suggested division by fission but this was never verified.

12. Strain Ri, 48 hours. Note multiple filamentation at upper left. Just below is a zooglee-like mass showing apparent fragmentation of filaments.

13. Strain Russ, 48 hours. The largest form, near top center, was not duplicated in entirety by any other strain but forms consisting of 1 or 2 such segments were noted in all strains.

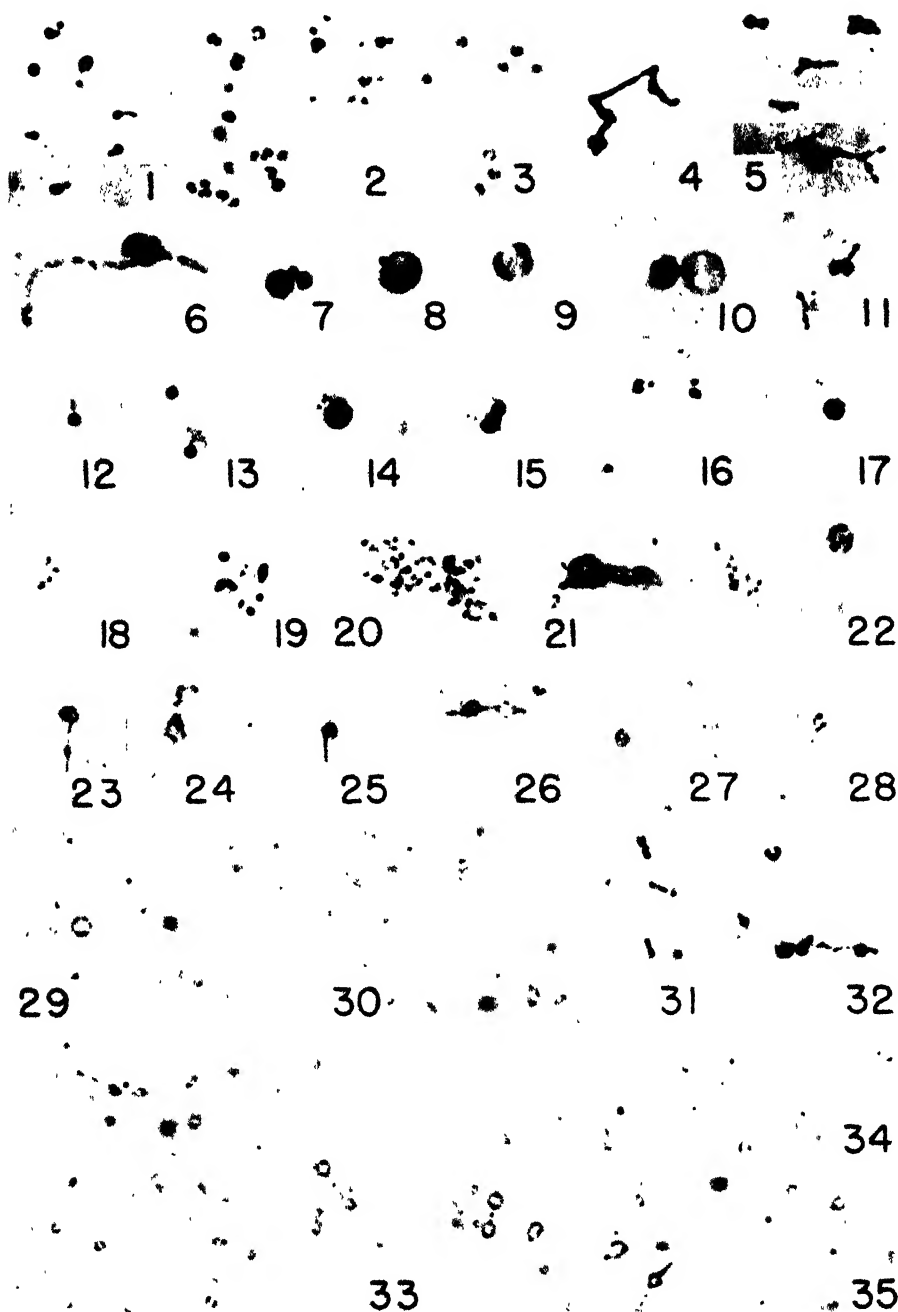


PLATE III

Photomicrographs of supravital stained organisms. Reproduced with slight reduction from original magnifications of 2,250 diameters.

1. Chri and Memp strains. Hofmann's violet and aqueous gentian violet stains. Sessile and filamentous budding. In gelatin hydrolyzate medium (Gel. HOH).

2. Left: Russ strain. Hofmann's violet in saline suspension from solid medium. Coccoid forms.  
Right: Memp strain. Hofmann's violet, in Gel. HOH medium. Coccoid forms; filamented and sessile buds.
3. Memp strain. Hofmann's violet, in Gel. HOH. Coccoid forms.
4. 38 strain. Hofmann's violet, in saline suspension. Bizarre chain due to budding.
5. 1. Russ strain. Hofmann's violet, in saline suspension. Multiple budding.  
2. Memp strain. Safranin O, in Gel. HOH. Budding producing branching.  
3. Same. Bipolar staining bacillary form.  
4. 38 strain. Bismarck brown Y, in saline suspension. Branched filament.
6. Memp strain. Bismarck brown Y, in Gel. HOH. Globus attached to a chain of coccoid and bacillary forms. Which gave origin to the other is not known. Globus shows heavy mass of chromatin at part opposite attachment site. Also within are 2 bodies of usual coccoid size. Suggests sporangium formation with 2 sporangiospores as usual number. (Cf 29 in plate II, also 18 in plate I. See also 10 below.)
7. Russ strain. Hofmann's violet in saline suspension. Primary and secondary budding from a smaller globule. The globule also contains 2 bodies of usual coccoid dimensions. Note cell wall of secondary bud.
8. 38 strain. Bismarck brown Y, in saline suspension. Large globule with sessile bud.
9. 38 strain. Hofmann's violet, in saline suspension. Large globule. Note characteristic peripheral distribution of chromatin.
10. Schu strain. Bismarck brown Y, in saline suspension. Globus with globular bud on a short filament. Note 2 additional potential budding sites in perimeter of globus. The daughter globule contains 2 bodies of coccoid dimensions, also additional peripherally located chromatin.
11. 38 strain. Bismarck brown Y, in saline suspension. Twinned coccoid forms, one filamented. Note M.R.U. in filament.
12. Memp strain. Hofmann's violet in Gel. HOH medium. Coccoid form of usual size with very slender short filament containing 2 M.R.U.
13. Russ strain. Safranin O, in saline suspension. Small coccoid form with multi-branched filament. Note the M.R.U. at tips of fine branches.
14. Russ strain. Hofmann's violet, in saline suspension. A 2- $\mu$  globule showing part of a meridional budding fringe.
15. Same. Peripheral filamentous budding. Note the M.R.U. along the filaments.
16. Memp strain. Hofmann's violet, in Gel. HOH. Coccoid forms, one showing a filamented bud.
17. Schu strain. Hofmann's violet, in Gel. HOH. Globule with chromatin chiefly in granules at periphery.
18. Russ strain. Hofmann's violet, in saline suspension. Clump of filamentously connected M.R.U., some developing toward usual coccoid form size.
19. Same. Development of M.R.U. to larger forms. Note delicate filaments.
20. Memp strain. Hofmann's violet, in Gel. HOH. Clump of developing M.R.U.
21. Same. Ruptured globus. Parachutelike arrangement of delicate filaments, each connected to one or more minimal reproductive units.
22. Russ strain. Hofmann's violet, in saline suspension. Oval globule with peripherally distributed chromatin.
23. 38 strain. Bismarck brown Y, in saline suspension. Filamented coccoid form. Note peripherally distributed chromatin, and M.R.U. along course of filament.
24. Russ strain. Bismarck brown Y, in saline suspension. Globule with sessile bud. Also twinned coccoid forms, one filamented with a M.R.U. at tip.
25. Memp strain. Bismarck brown Y, in Gel. HOH. Filamented coccoid form showing delicate cell wall.
26. Russ strain. Bismarck brown Y, in saline suspension. Filamentous linkage between well-stained, living coccoid form and a lightly and unevenly stained, degenerating one.

## OBSERVATIONS OF SUPRAVITALLY STAINED ORGANISMS

Supravital staining greatly enhanced visualization of all structural details and permitted better photographic registration than we could secure otherwise. Nuclear particles were stained within 10 minutes by most dyes and were seen to be typically peripheral in location and often multiple. Minute buds stained blue with Nile blue sulphate, and filaments stained faintly. Under the dark field the buds had a deep lilac-rose color. Hofmann's violet stained chromatin a deep violet color; minute buds and filaments a light violet. Under the dark field chromatin particles appeared red and yellow; the minute buds always a clear yellow. Malachite green also showed differential staining between nuclear chromatin and the minute buds, the former taking a clear green and the latter a terra cotta or brick red. Filaments stained poorly. Bismarck brown Y stained all structural parts including delicate cell walls and the finest filaments. Coccoid and bacillary forms, globules, globi, thick and thin filaments, and what appeared to be minimal reproductive units were readily visualized as demonstrated in plate III.

The chromatinlike particles within the coccoid forms were usually ring-shaped, discoidal, navicular, or crescentically obovoid, and almost always peripherally located, as noted previously. An extremely delicate, spheroidal, veillike cell wall surrounded these forms, best seen when the diameters reached or exceeded  $0.5\ \mu$ .

In larger globoid forms nuclear chromatin was often dispersed into peripheral granules. The equatorial or meridional lines of granules around the circumferences of the larger globules sometimes gave origin to very short delicate filaments, each with a minute minimal reproductive unit as its free end, the entire rows undulating in harmonic wave motion and giving the appearance of budding fringes. Occasionally filaments were seen within globules; some forked or branched, others unbranched and coiled within the cell walls. Many of these internally located filaments were examined critically, and their cylindrical shape was repeatedly confirmed. However, it is quite possible that these structures

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Two fine filaments spring from living unit, each with a M.R.U. at tip. Chromatin peripheral.

27. Memp strain. Bismarck brown Y, in Gel. HOH. Coccoid form with long slender filament with a M.R.U. at tip. Shows delicate cell wall and peripheral chromatin granules.
28. Same. Filamented coccoid form with M.R.U. in filament.
29. Same. Globular and bacillary forms. Note minute spherule at tip of filament.
30. Same. Coccoid and bacillary forms. Note M.R.U. in filament.
31. Schu strain. Hofmann's violet, in Gel. HOH. Bacillary forms with unevenly distributed chromatin.
32. Russ strain. Bismarck brown Y, in saline suspension. Filamentously connected coccoid forms with M.R.U. within filament.
33. Memp strain. Bismarck brown Y, in Gel. HOH. Coccoid and bacillary forms; some filamented with M.R.U. at tips of filaments.
34. Same. Coccoid and bacillary forms.
35. Same. Globules and dumbbell and coccoid forms, one filamented. Note peripherally distributed chromatin particles and minimal reproductive units within filaments.

may expand and eventually become globoid masses, thus accounting for the two sporangiosporelike bodies so often seen within globi.

Sessile and filamentous budding were observed. Chains of coccoid or bacillary forms, or chains composed of irregular mixtures of these forms, occasionally showed considerable branching from one or more nodal budding sites.

Minimal reproductive units were observed as suspended, discrete particles, at the termini of long or short filaments, and at various locations along the courses of filaments. Some small coccoid forms gave origin to a single short filament, which branched into a cluster of fine terminal filaments, each with a minute unit at its tip. These minute units were formed within or on filaments produced by coccoid, bacillary, or globular forms.

#### OBSERVATIONS OF INFECTED HEART BLOODS

Examination of infected heart bloods revealed with certainty only the small coccoid forms. The suspended minute particles in shed blood, the presence of degenerating blood cells, and filamentous structures derived from various cells made recognition of other microorganismal forms uncertain.

#### MODES OF REPRODUCTION OF BACTERIUM TULARENSE

Although we made frequent drawings of several strains at successive stages of growth, we could not consistently demonstrate a predominant morphologic phase at any time during the first 8 days. Even with very small inocula in gelatin hydrolyzate media it seemed that as soon as there were 15 to 20 organisms to the oil-immersion dark field a prolonged search would reveal at least one example of every form yet observed. Certain gross trends were noted. Large globi seldom developed before the third or fourth day unless the culture was incubated in an atmosphere of pure oxygen. Filaments, filamentous budding, and complex filamented chains seldom appeared before the third or fourth days. Early growth at room temperatures (24 to 26 C) was chiefly bacillary or mycelial in nature with frequent sessile budding. Filaments and filamentous budding appeared only after several days of growth, and were not frequent until the seventh day.

Figure 2 illustrates the trend of morphologic changes during the first week of cultivation in gelatin hydrolyzate medium of strain Memp. All tubes contained 5 ml of medium. The tubes for incubation at 37 C and at room temperature were each inoculated with 0.10 ml of a 24-hour liquid culture. The effect of an oxygen atmosphere was tested by inoculating similarly 7 other tubes of media, displacing air by pure filtered oxygen, pushing down the cotton plug, and sealing tube tops with parafilm. These tubes were incubated at 37 C. One tube was opened each day and swirled gently to resuspend any sediment; examinations were made of sealed cover slip preparations under dark-field illumination.

At no time during the two-year period of study could we demonstrate reproduction by binary fission. Since slide microcultures were seldom wholly satisfactory, possibly owing to obligate aerobic requirements of the organism, observations on modes of reproduction were necessarily limited to intermittent observations of cultures in gelatin hydrolyzate media.

Multiplication by budding was observed frequently, and this appeared to be the chief mode of reproduction. Both sessile and filamented buds were produced by bacillary forms, by refractile round filamentous forms, and by coccoid forms of all diameters from  $0.45\ \mu$  to the large globi of 3 to  $3.5\ \mu$ . Buds often appeared on the cell walls at the sites of the clear circular areas previously described, often at  $120^\circ$  of arc apart. They developed into coccoid forms of the usual size or, occasionally, into short or long bacillary forms. Chains of coccoid forms occasionally developed from a single budding site. Some chains were formed by

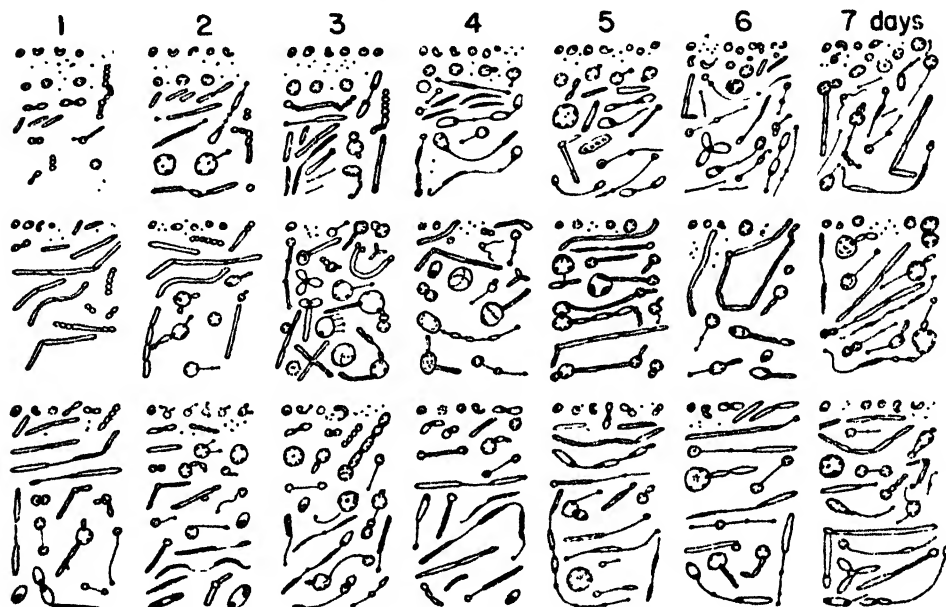


FIG. 2. Illustrates certain developmental trends of strain Memp in gelatin hydrolyzate medium during the first 7 days of cultivation. The upper row represents observations of a culture incubated at  $37^\circ\text{C}$ . Small coccoid forms outnumbered all others. Filaments, filamented forms, and globules were frequent only after the fourth day. The middle row is from a similar culture incubated at  $24$  to  $26^\circ\text{C}$ . Mycelial or bacillary forms predominated until the fourth day. Budding from these forms was frequent. Globules appeared on the third day. The lower row represents growth under an atmosphere of oxygen. Growth was heaviest in these tubes and predominantly the small coccoid form for 5 days. Thereafter bacillary forms and long filamented chains showed a marked increase. Sessile budding was frequent after the third day. Highly refractile bacillary forms capable of flexional movements are indicated by solid black.

continuous sessile budding; others developed with filamentous linkage between members. Chains seldom exceeded a total of 4 or 5 units unless they were of the filamented type. From a small globus we saw terminal nonfilamentously chained coccoid forms break off, singly and in pairs. Thereafter the same globus sprouted two more buds at new sites about  $120^\circ$  of arc apart and resumed formation of nonfilamented coccoid chains. There was considerable protoplasmic commotion within the cell wall at the budding site, and six or seven rapid, outpouching, and retracting movements were made through the cell wall before the bud finally remained outside. This process was repeated for each additional coccoid

form as it was added to the chain. Twenty to 30 minutes elapsed between delivery of each new bud. The protoplasm of the globus appeared to be consumed by the new forms, or converted entirely into them. After cessation of budding the residual globus consisted only of a delicate, lightly refractile, spheroidal, shrunken cell wall entirely devoid of refractile contents. We observed several examples of this type of budding from globoid forms of 1.5 to 3  $\mu$  in diameter.

One example of filamentous budding was observed. The globus was about 3  $\mu$  in diameter. It added a minute, solid, spherical, coccoid form, by extrusion, to a long filamented chain that already consisted of two 0.5  $\mu$  coccoid forms, a long bacillary form, and a long terminal filament. Here, again, it required 5 or 6 intermittent, vigorous, outpouching or extrusive movements through the cell wall at the site of junction with the fine filament, each outthrust apparently surrounding and enclosing the proximal end of the filament before the new spherule was delivered to a permanent place on the filamented chain.

Formation of fine filaments was not observed despite prolonged anticipatory search. Marked organisms without filaments were noted at later intervals to possess one or more of them, but the act of formation was not seen. This was true also for the development of minimal reproductive units. Filaments without them were noted later to have one or more minute condensed areas along their lengths or at their tips. Further observations demonstrated clearly that the filaments increased slowly in length, and that the minimal reproductive units enlarged to form spherules, coccoid forms, bacillary forms, and even large globi. This was true both for filaments that had and had not been broken off by Brownian movement from the generative form. It appeared almost certain that separation of coccoid or bacillary forms, due to forces exerted by Brownian motion or by manipulation of cultures, resulted in formation of filaments. Most long filaments are undoubtedly so formed, and it is possible that all are made in this manner. Both organisms and filaments adhere readily to glass surfaces.

Formation of minimal reproductive units was inferred from prolonged intermittent observations of living cultures. Though they lack the continuity that is necessary to establish certainty, we regard our serial drawings, collected in figure 3, as a reasonably good approximation of events. The figure also represents progressive development of minimal reproductive units into larger forms.

One variety of small spheroidal forms always had a dense, central, highly refractile body that stained solidly and deeply with the supravital dyes. Further development of this form always resulted in radial peripheral budding, as indicated in the upper and next to bottom rows of the right-hand column in figure 3. One is also shown at the bottom of 29 in plate II. The development of this uncommon, centrally nucleated form was observed in liquid media as well as in one series of slide microcultures stained at intervals with Bismarck brown Y.

The forms shown at 18 and 22 in plate I, the middle form of 29 in plate II, and 6 and 10 in plate III, all consist of globi containing 2 densely staining coccoid or ovoid bodies about 0.5  $\mu$  in diameter. Such globi may or may not possess additional chromatin material. The forms shown in plates II and III strongly



suggest the appearance of sporangia, each containing two sporangiospores. The one photographed and shown as 6 in plate III was studied for a long time, but it was not possible to be sure whether the globus had budded from the chain of coccoid forms or the chain had been produced by the globus. Nor were we able to determine this relation in other examples. The number of enclosed bodies was always two.

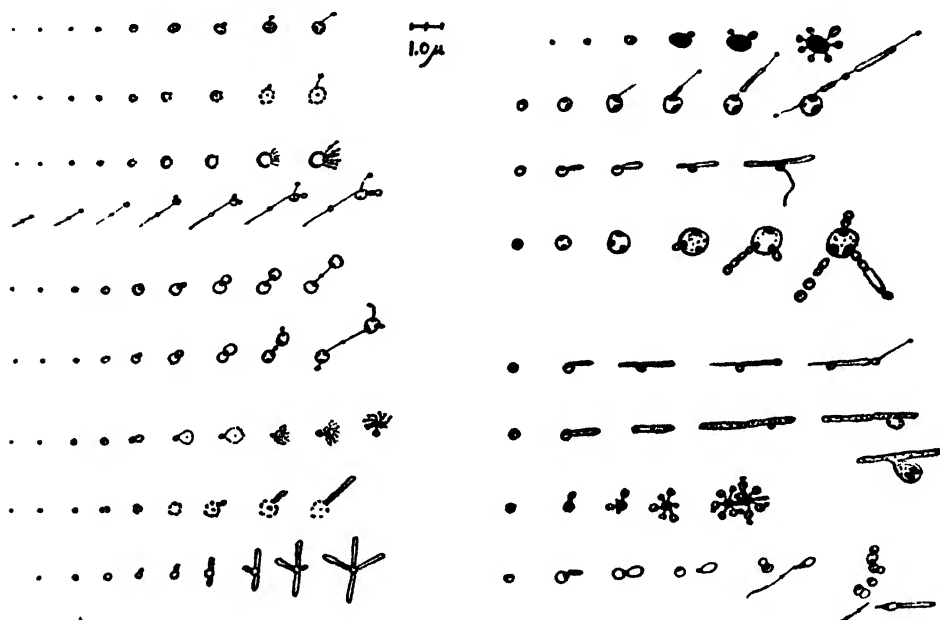


FIG. 3. The left-hand column illustrates progressive development of various forms from minimal reproductive units as inferred from many series of intermittent observations. It also indicates certain modes of production of these minute units. Development of additional forms from the predominant  $0.45\mu$  coccoid form is shown at the right. The suggested development of the sporangiumlike body, in the sixth row, is purely conjectural and possibly incorrect. All other drawings were based on actual observations. In all cultures it seemed that these various modes of reproduction were operative concurrently.

These preliminary studies indicate that *Bacterium tularensis* is "polygenethodic," possessing multiple modes of reproduction.

#### DISCUSSION

As knowledge of natural and experimental tularemia, and information about the causative agent, accumulated during recent decades it became increasingly apparent that the concept of *Bacterium tularensis* as an ordinary bacterium, classified usually as *Pasteurella tularensis*, was inadequate to account for the known facts and relations. The organism has obligate requirements for cystine and for an unidentified protein derivative and, until recently, was incapable of multiplication in any liquid medium. Filtration experiments with tested earthenware filters showed that about half of all filtrates were infective for rodents and that no exotoxin was formed. The agent passes the normal unbroken skin

or mucosa, and has a high degree of virulence or infectivity. Light suspensions of virulent strains, of usual agglutination antigen turbidity, will sometimes kill 20 to 25 per cent of mice or guinea pigs in dosage of 0.5 ml of a  $10^{-12}$  dilution. The number of natural animal hosts is large, including many rodents, birds, reptiles, amphibia, felines, canines, and marsupials. The number of insect and arthropod hosts and vectors is large. Steinhaus (1942) lists 52 insects and arthropods associated in some manner with this organism. Three ticks are good biologic hosts, transmitting the organism via their eggs, larvae, and nymphs to adults of the next generation. It is further transmitted among adults by copulation. Within ticks the organism grows both in extra- and intracellular locations. In rodents intracellular multiplication also occurs; it is best seen in the hepatic cells of the mouse where large pseudocysts are eventually formed, but it is also noted in hepatic cells of the hamster and guinea pig, in pulmonary macrophages of several rodents, and in splenic vascular endothelium and lymph node reticular cells of mice. The disease is highly fatal for most rodents, but causes low mortality and prolonged morbidity in man, leaving recovered individuals with an amazingly solid immunity against subsequent exposures. Serum agglutinins acquired by infection persist for the remainder of life of the recovered patient. Practically all attempts to provoke active immunity in rodents with light and dense suspensions of bacteria killed by various means have failed to demonstrate any notable resistance to experimental infection against a single M.L.D. of a highly virulent strain. Similarly, hyperimmune sera produced in large animals by inoculation of killed or living cultures of titred high virulence have consistently failed to protect mice or guinea pigs against a single M.L.D. of a challenge strain of high virulence.

Knowledge of the morphology of the organism offers possible solutions to only some of the problems raised by the above statements. Nevertheless, the findings presented here, easy to confirm by simple methods of study, emphasize anew the necessity for reorientation of concepts concerning tularemia and the ecologic and biologic characters of its causative agent.

Since the morphologic units of *Bacterium tularense* consist of coccoid and cylindrical bacillary forms, flattened bacillary forms, globules and globi, filaments, and minimal reproductive units, and since the organism possesses several modes of reproduction and multiplies in cell-free media, but not in media devoid of protein or protein derivatives, this microorganism satisfies most of the criteria for classification in the pleuropneumonia group. Satisfactory evidence for filtrability of the minimal reproductive units, size of particles, and developmental forms of these units in animals and in culture media is not offered here. Some evidence that has been obtained will be presented separately. Examination of our photomicrographs and drawings will demonstrate that certain forms of *Bacterium tularense* bear striking resemblances to various organisms of the pleuropneumonia group as they are depicted in studies by Ledingham (1933), Klieneberger (1934), Turner (1935), and Sabin (1941). Our study of a recently isolated strain of *Streptobacillus moniliformis* by similar methods showed some forms which we could not differentiate morphologically from certain *Bacterium*

*tularensis* forms. The L<sub>1</sub> microorganism derived from this culture resembled *Bacterium tularensis* more closely. Similar resemblances are also apparent in the photomicrographs by Brown and Nunemaker (1942). The similarities are only partial, existing for only certain forms of these highly polymorphous microorganisms. Our plates also demonstrate morphologic features apparently unique for *Bacterium tularensis*.

Although these studies indicate that *Bacterium tularensis* should not be included in the *Pasteurella* or *Brucella* genera, we are not proposing a new generic name for it. We believe it is more closely related to the pleuropneumonia group than to any other group clearly defined at present, but that certain of its apparently unique morphologic features, herein described and photographed, set it apart from this group as it is now constituted. We think that creation of a new generic name now would be premature and based upon insufficient knowledge of this group and its relatives. We suggest postponement until these relations can be more clearly defined. Until then we believe that the more noncommittal name, *Bacterium tularensis*, is the one of choice and that *Pasteurella tularensis*, *Brucella tularensis*, and *Coccobacterium tularensis* should be declared invalid. We further suggest that when an appropriate generic name is adopted, the proper gender form of *tularensis* be retained as the specific name.

#### SUMMARY

*Bacterium tularensis* is an extremely polymorphous microorganism. It possesses neither capsules nor flagella, and is nonmotile. Its morphologic units include globi and globules, flat and cylindrical bacillary forms, coccoid forms, delicate filaments, and minimal reproductive units. Many forms, under certain conditions of staining, reveal prominent, thick cell walls. The cell wall is, of course, an integral structural unit with no relation to degree of invasiveness or virulence. All 43 strains were morphologically identical. We found no morphologic feature to differentiate a virulent from a nonvirulent strain. All strains seemed to possess multiple modes of reproduction, and budding was apparently the chief one. Division by binary fission was not observed, though it may occur. Although the organism seems to be related to the pleuropneumonia group, perhaps only distantly related, we suggest postponement of taxonomic efforts until the members and relations of this group are better defined. For the present we urge invalidation of the terms, *Pasteurella tularensis*, *Brucella tularensis*, and *Coccobacterium tularensis*, and retention of *Bacterium tularensis* as the most suitable temporary designation.

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# SOME OBSERVATIONS ON THE FILTRABILITY OF BACTERIUM TULARENSE

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Throughout our morphologic study of *Bacterium tularense* in liquid medium cultures we noted constantly the presence of very minute forms (Hesselbrock and Foshay, 1945). These appeared to grade downward in size from the most prevalent coccoid or ring forms of about  $0.5\ \mu$  in diameter to bodies so small that neither size nor shape was discernible. Such particles were never seen in uninoculated media, either before incubation or for 96 hours thereafter. Some particles were discrete and freely suspended; others were attached by delicate short filaments to larger morphologic units. Many additional ones were seen lying in, and apparently arising within, delicate filaments of variable length. The number of discrete particles was never large at any time in any of the many cultures that were examined. Single particles in suspension, as well as those filamentously attached or interconnected, stained a brick-red color when supravirtally stained with 2 per cent aqueous malachite green, whereas all large morphologic units stained green. None of six other common pathogenic bacteria, cultivable in the same medium, produced particles of this size. Prolonged intermittent observations of cultures, although inadequate to establish the fact, suggested strongly that these particles were capable of reproducing all other forms of this pleomorphic organism and, apparently by means of larger units, of reproducing themselves. Our provisional interpretation was that they represented true morphologic units of *Bacterium tularense*, possibly to some degree analogous in size and function to the minute elementary bodies formed by organisms of the pleuropneumonia group, appropriately called minimal reproductive units, M.R.U. (Sabin, 1941).

It has long been known that virulent strains of the organism sometimes, in about one half the recorded trials, pass Berkefeld, Chamberland, and Seitz filters. We cannot find reports on the morphology of the units that passed the filters, but our observations suggested that the provisionally designated M.R.U. might have accounted for the infectivity of the filtrates. To test our views we used gradocol membranes for ultrafiltration of virulent cultures.

## MATERIALS AND METHODS

Two equally virulent strains were used. Throughout the 21 months during which filtrations were spaced, frequent titrations in white mice gave LD<sub>50</sub> titers between 9.3 and 9.5. Infrequent tests in rabbits, using suspensions of T-860  $\times 10^{-8}$  dilution (initial turbidity equivalent to that of the standard U.S.P.H.S.

<sup>1</sup> In partial fulfillment of the thesis requirements for the degree of Doctor of Philosophy.

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agglutination test suspension), killed all animals within 5 days. Two filtrations were performed after cultivation for 48 and 72 hours, respectively, in a gelatin-hydrolyzate glycerol cystine liquid medium. The third filtration was performed with a pooled saline suspension of growths from 4-, 5-, and 6-day cultures on blood glucose glycerol cystine agar. All initial turbid suspensions were cleared by centrifugation before passing the supernatants through the preliminary screening membranes.

Original Elford gradocol membranes were used with low positive nitrogen pressure. Immediately prior to filtration all membranes were satisfied by passing about 12 ml of beef heart infusion broth of pH 7.7. The entire culture supernatant was then filtered through a coarse membrane. The first liquid culture and the solid media culture supernatants were screened through membranes of 900 m $\mu$  A.P.D. After removing samples the filtrates were divided, and equal portions were simultaneously filtered through membranes of 600, 500, 400, and 300 m $\mu$  A.P.D. The second liquid culture supernatant was screened through a 770 m $\mu$  A.P.D. membrane, then treated as were the others. The major portion of each filtrate was obtained by gravity filtration. Pressures of 2 lb/sq in were applied until filtration ceased; then terminal pressures of 10 lb/sq in were applied to ensure completion. Inspection of all membranes after filtration revealed no defects.

Samples of each filtrate were treated as follows: Large loopfuls were examined by dark-field illumination; from 0.20 to 1.0 ml amounts were inoculated into duplicate series of gelatin hydrolyzate medium; large loopfuls were transferred to slants of blood glucose glycerol cystine agar in duplicate; either 0.5 or 1.0 ml was inoculated intraperitoneally into groups of 5 or 10 mice each; and from 0.1 to 1.0 ml was cultured on 20 per cent serum agar and in 20 and 30 per cent serum broths. Two preliminary screening filtrates, one each from 900 m $\mu$  and 770 m $\mu$  A.P.D. membranes, were titrated for numbers of infective units by intraperitoneal inoculation into mice of 0.5 ml quantities of ascending twofold dilutions.

Necropsies were performed on all mice that died. Heart blood cultures were made and a portion of each liver was removed for histologic study. The constancy with which this disease produces characteristic lesions in the mouse liver, hepatic cell pseudocysts filled with bacteria, and typical foci of necrosis makes this organ an extremely reliable one for detection and verification of tularemic infection.

#### EXPERIMENTAL OBSERVATIONS

*Solid medium cultures.* The supernatant of pooled solid medium cultures gave evidence of passing only the 900 m $\mu$  A.P.D. membrane. All mice inoculated with this filtrate died on the third day; they yielded heart blood cultures pure for *Bacterium tularensis*, and liver sections that showed typical tularemic lesions. Cultures were readily obtained from solid and liquid media that contained cystine. All cystine-free media remained sterile despite careful search for pleuropneumonia-like organisms. The filtrate was unexpectedly rich in infective units, killing all mice in our highest dilution so that an end point was not secured.

Filtrates from 600, 500, and 400  $m\mu$  A.P.D. membranes remained sterile after cultivation in all media, and failed to kill any mouse of lots of 10 each, inoculated intraperitoneally in 1.0 ml amounts.

*Liquid medium cultures.* The culture supernatant that was screened through a 900  $m\mu$  A.P.D. membrane yielded infective filtrates from the 900 and 600  $m\mu$  A.P.D. membranes but not from membranes of lesser A.P.D. Discrete particles were observed by dark-field illumination only in the two filtrates that later proved to be infective. With the minimal inocula stated above, all filtrate cultures remained sterile. The 900  $m\mu$  A.P.D. membrane filtrate was not titrated for infectivity. All of 10 mice inoculated with 0.5 ml each of this filtrate died; they yielded heart blood cultures positive for *Bacterium tularense*, and liver sections with typical tularemic lesions. Of 10 mice each inoculated with 0.5 ml of the 600  $m\mu$  A.P.D. membrane filtrate, 4 died with evidences of tularemia. Two of these gave heart blood cultures pure for *Bacterium tularense*, and liver sections that were typical for this infection. The other two gave heart blood cultures positive for *Salmonella*, and liver sections characteristic for "mouse typhoid" but with a few tularemic lesions also. Of the remaining 6 mice, those that died were shown by cultures and sections to have died of *Salmonella* infection. Neither cultural nor histologic evidence of tularemia was found for any dead mouse inoculated with a filtrate from a membrane with an A.P.D. smaller than 600  $m\mu$ .

The culture supernatant that was screened through a 770  $m\mu$  A.P.D. membrane yielded a cultivable and infective filtrate only from the 770  $m\mu$  A.P.D. membrane. Direct cultures grew *Bacterium tularense* but no other organism. All of 10 mice each inoculated with 0.5 ml of this filtrate died; they yielded heart blood cultures positive for *Bacterium tularense* only, and liver sections with characteristic tularemic lesions. Titration of this filtrate for infectivity, in serial twofold dilutions in lots of 5 mice each, revealed that the 1:4 dilution killed exactly 50 per cent of the mice. Except for one death by accidental trauma, all mice, 10 in each lot, survived inoculation with filtrates from membranes of 600, 500, 400, and 300  $m\mu$  A.P.D.

*Morphology of cultures derived from filtrable forms.* The two cultures derived from heart bloods of mice inoculated with filtrate from a membrane of 600  $m\mu$  A.P.D. were subjected to morphologic study by methods previously described. Each culture developed all morphologic units previously found to be constant and characteristic for the species—cocci and ring forms, flattened and rounded bacillary forms, globules, filaments, and minimal reproductive units both solitary and filamentously attached and interconnected (Hesselbrock and Foshay, 1945).

*Pathologic changes in livers of white mice.* When mice are fatally infected by the ocular or cutaneous routes with whole cultures or with infected tissues, or by subcutaneous or intraperitoneal injections of moderate to large inocula of culture suspensions, the characteristic pathologic changes in the livers are (1) numerous foci of necrosis with extensive karyorrhexis and (2) a small to moderate number of pseudocysts, swollen hepatic cells completely filled with *Bacterium tularense*.

All mice fatally infected by membrane filtrates showed a reversed ratio of



these hepatic features, numerous large pseudocysts and few to moderate numbers of necrotic foci. This remarkable alteration in liver lesions was not ascribable to the nature or size of the infecting morphologic unit but probably to the number since identical changes were consistently produced by inocula of suspensions of whole cultures in serial decimal dilutions from  $10^{-6}$  upwards.

#### DISCUSSION

Our original aim to determine critically the dimensional limits of the M.R.U. remains unfulfilled and is reluctantly postponed due to current conditions of work. Nevertheless it was demonstrated that morphologic units that passed a 600  $m\mu$  membrane but not a 500  $m\mu$  membrane, and hence were in the range of 300 to 350  $m\mu$  in diameter, were infectious, and that their development resulted in the complete series of morphologic units now known to be characteristic for *Bacterium tularensis*. At no time, from either cultural or animal sources, were we able to demonstrate the presence of any associated or concomitant microorganism other than the easily identified "contaminants" from mice.

Since 0.5 ml of a 1:4 dilution of a 770  $m\mu$  membrane filtrate was the LD<sub>50</sub> dose, it is apparent that our cultural conditions did not yield filtrates rich in M.R.U. A true estimate of the size of the smallest units will require filtrates containing many more M.R.U. Since a large proportion of M.R.U. observed at any time during 8 days of cultivation in gelatin hydrolyzate medium was always found to be within filaments, or filamentously attached to larger morphologic units, the cultural needs are those that will yield a high proportion of discrete particles of the smallest size. Knowledge of the rate of development of M.R.U. to larger units is also desirable.

#### SUMMARY

The filtrable form of *Bacterium tularensis* in gradocol membrane filtrates of gelatin hydrolyzate medium cultures of highly virulent strains produced tularemia in the highly susceptible mouse and was recoverable in the form of larger morphologic units by heart blood cultures. These filtrable forms were approximately 300 to 350  $m\mu$  in diameter. We are reasonably sure that minimal reproductive units of smaller size exist, and that accurate measurement will require better cultural conditions than those hitherto obtained. Morphologic studies of cultures obtained from mice inoculated with filtrates demonstrated that the filtrable units were capable of development into all other morphologic units known for this organism.

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# THE INCREASE OF BACTERIOPHAGE IN VIVO DURING EXPERIMENTAL INFECTIONS WITH *SHIGELLA PARADYSENTERIAE*, FLEXNER, IN MICE

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The results obtained by Morton and Engley (1944) on the protective action of dysentery-phage against experimentally induced infections in white mice with *Shigella paradysenteriae*, variety Flexner, demonstrated that the experimental animals were protected when the ratio of dysentery-phage particles to virulent dysentery bacilli was of the order of 1 to 8. If the protective action of dysentery-phage *in vivo* is due to lysis of the dysentery bacilli by the dysentery-phage, the protective action is difficult to explain unless the amount of dysentery-phage within the animal's body increases during the course of the infection. Our results show the latter does occur.

During the course of our studies two reports appeared which corroborated our findings. Rakieta and Rakieta (1943) reported that the survival of chick embryos infected with *Shigella paradysenteriae*, Flexner, was associated with an increase in the amount of dysentery-phage in the embryos. Dubos, Straus, and Pierce (1943), working with *Shigella dysenteriae* infections in mice, demonstrated an increase in the amount of dysentery-phage in the infected mice.

Krueger and Scribner (1941) interpreted a portion of the results reported by Nungester and Watrous (1934) as indicating that 4 hours after the injection of bacteriophage into the animal body only 0.04 per cent remained in the circulating blood. Only 1 out of 4 rats showed the presence of bacteriophage in its blood, and Krueger and Scribner took one-fourth of the titer of the one rat for the basis of their statement on the rapid elimination of bacteriophage from the animal body. Such a general statement cannot be made on the basis of so few experimental animals.

The purposes of our investigations were to determine (1) how rapidly our strain of dysentery-phage was eliminated from the circulating blood of white mice and (2) if there was an increase in the amount of dysentery-phage in the blood of the experimental animals during the course of infection with dysentery bacilli. We found that the dysentery-phage gradually disappears from the circulating blood within a period of about 1 week. During the course of infection with phage-susceptible dysentery bacilli, the titer of dysentery-phage markedly increases in the blood of the experimental animals.

## EXPERIMENTAL

In the experiments to be described cultures of *Shigella paradysenteriae*, varieties Flexner X-S45 and Newcastle, were used to produce the experimental infections.

Young white Swiss mice weighing 17 to 20 grams were used as experimental animals. The intraperitoneal injection into mice of the organisms suspended in 5 per cent gastric mucin produces in a few hours a bacteremia which terminates fatally in 24 to 48 hours. When suspended in 5 per cent gastric mucin, 4 bacilli constitute a minimum lethal dose.

The bacteriophage preparation used was propagated for several generations against cultures of the X-S45 organism. Filtrates were obtained as follows: About 150 ml of tryptone glucose yeast-extract broth were placed in a 250-ml centrifuge bottle and inoculated with 2 ml of an overnight culture of the X-S45 organism. The bottle was then placed in a 37 C water bath for 2 hours, air being bubbled constantly through the growing culture. After 2 hours of incubation the culture was removed from the water bath and inoculated with 2 ml of the stock homologous bacteriophage. The culture was then replaced in the water bath and kept there for 5 hours, air being bubbled through it constantly. The bottle was then removed from the bath and centrifuged at about 3,500 rpm for 20 minutes. The supernatant was filtered through fritted pyrex glass filters and the filtrate aseptically dispensed into bottles and placed immediately in the refrigerator. The titer of each lysate was determined 24 to 48 hours after preparation by plaque enumeration on 2 per cent extract agar plates. The plaques were counted after overnight incubation of the plates at 37 C. The filtrates used in the experiments were: LP X-S45 no. 6 ( $5 \times 10^{10}$  particles per ml), LP X-S45 no. 7 ( $1.4 \times 10^9$  particles per ml), and LP X-S45 no. 8 ( $4.8 \times 10^9$  particles per ml).

The blood level of the bacteriophage in normal and infected animals was determined by bleeding from the heart, using  $\frac{7}{8}$ -inch, no. 23 or 24 gauge needles and 1-ml (tuberculin) syringes. The syringes and needles were sterilized in boiling water and were allowed to dry before use.

Either 0.1 or 0.2 ml of blood were withdrawn from the heart, and initial dilution of 1/10 was prepared in a young broth culture of the "X" organism. Appropriate dilutions were made and 0.2 ml of each dilution were placed on the surface of an agar plate and spread with a sterile, bent glass rod. (Dilutions were made with 1-ml pipettes, a different pipette being used for each dilution.) The plates were incubated overnight and examined the following day or placed in the refrigerator after incubation and examined within the next 48 hours. When very small amounts of phage were to be expected, sometimes as much as 0.5 ml of each dilution was spread on the surface of the agar plate.

*Experiment 1.* A group of 18 mice received intraperitoneally 1 ml of a culture of the X-S45 organism diluted in gastric mucin. The actual amount injected was 0.2 ml of a  $10^{-7}$  dilution of the culture suspended in 0.8 ml of mucin. Simultaneously the mice were given 1 ml of a homologous phage ( $5 \times 10^{10}$  particles) intraperitoneally. At the same time an equal number of mice received the injection of dysentery-phage only. (All of the 3 normal mice which received the injection of organisms only were dead within 24 hours.)

An equal number (usually 3) of both the infected and the control mice were bled at intervals of 3, 9, 24, 48, 72, and 96 hours after receiving the injections.

The blood level was determined and the average for each group was obtained. The results are tabulated in table 1.

The results of this preliminary experiment, shown in table 1, give only slight indication of multiplication of bacteriophage *in vivo* among the infected animals. There is some indication, however, that the phage increased in amount and was retained for a longer period of time in the blood of the infected animals. The appearance at the 3-hour interval of less dysentery-phage in the circulating blood of the infected mice as compared with the normal mice suggested that the dysentery-phage may have been fixed by the dysentery bacilli in the peritoneal cavity. The following experiment was devised to determine if this occurred.

*Experiment 2.* Forty-eight white mice, weighing 17 to 20 grams each, were given  $1 \times 10^9$  particles of dysentery-phage intraperitoneally. Another 6 mice were kept as normals. The titer of the dysentery-phage in the blood was determined (as previously outlined) 24, 48, 72, and 96 hours after the injection of dysentery-phage. At the 96-hour interval 9 mice were injected intraperitoneally each with 127 M.L.D. of the X-S45 organism in 5 per cent mucin; 9 mice received

TABLE 1

*Average number of dysentery-phage particles per ml of blood after the intraperitoneal injection of  $5 \times 10^{10}$  particles of dysentery-phage*

TIME INTERVAL AFTER INJECTION	AVERAGE PHAGE TITER FOR THE NORMAL MICE	AVERAGE PHAGE TITER FOR THE INFECTED MICE	NO. OF MICE BLED
<i>hours</i>			
3	$1.5 \times 10^3$	$9.0 \times 10^8$	3
9	$2.8 \times 10^8$	$4.5 \times 10^8$	3
24	$2.4 \times 10^8$	$3.9 \times 10^8$	3
48	$2.3 \times 10^8$	$1.0 \times 10^7$	3
72	$5.8 \times 10^3$	$4.0 \times 10^5$	2
96	$6.2 \times 10^2$	$5.0 \times 10^3$	2

mucin only, and 9 mice received 446 M.L.D. of a Newcastle strain of *Shigella paradysenteriae* not lysed by the dysentery-phage *in vitro*.

Three of the normal mice received 127 M.L.D. of the X-S45 organism and the remaining 3 received 446 M.L.D. of the Newcastle strain. These served as controls for virulence of the organisms. All 6 mice were dead within 24 hours. The results of this experiment are shown in table 2.

As may be seen from the data in table 2, the injection of the homologous organisms at a time when the dysentery-phage level in the blood had decreased to 200 particles per ml produced a rise in the blood level to an average of 16,000,000 particles per ml in 24 hours. This means that the number of particles had increased 80,000 times during this period. Seventy-two hours after the injection of the bacteria, the dysentery-phage level was still high, 5,300 particles per ml of blood.

On the other hand, in the group of mice which had received dysentery-phage but subsequently did not receive any organisms, the dysentery-phage level in

the blood decreased from the time of injection until it could no longer be detected with the dilutions employed after 144 hours, being less than 50 particles per ml.

The mice injected with mucin alone (96 hours after having received the injection of dysentery-phage) showed no increase in the number of particles in the blood. The average number of particles for each of the different groups of mice which received only mucin could not be determined since 5 of the total number of 9 mice showed less than 50 particles per ml of blood.

TABLE 2

*Blood level of dysentery-phage in control mice, in mice infected with phage-susceptible dysentery bacilli (X-S45), and in mice infected with dysentery bacilli not susceptible to the lytic action of the dysentery-phage (Newcastle strain)*

BLEEDING TIME AFTER INJECTION OF DYSENTERY- PHAGE	NUMBER OF DYSENTERY- PHAGE PARTICLES PER ML OF BLOOD IN MICE INJECTED WITH DYSENTERY-PHAGE ONLY	NUMBER OF DYSENTERY- PHAGE PARTICLES PER ML OF BLOOD IN MICE INJECTED WITH X-S45 ORGANISMS, 96 HOURS AFTER INJECTION OF PHAGE	NUMBER OF DYSENTERY- PHAGE PARTICLES PER ML OF BLOOD IN MICE INJECTED WITH NEW- CASTLE ORGANISMS, 96 HOURS AFTER INJECTION OF PHAGE	NUMBER OF DYSENTERY- PHAGE PARTICLES PER ML OF BLOOD IN MICE INJECTED WITH MUCIN, 96 HOURS AFTER INJEC- TION OF PHAGE
hr				
24	26,000,000 phage particles/ml blood			
48	(no results; wrong dilu- tions used)			
72	940			
96	200			
120	570	16,000,000	180	800 (1 mouse) <50 (2 mice)
144	50	11,800	all mice dead	150 (1 mouse) <50 (2 mice)
168	<50	5,300		100 (2 mice) <50 (1 mouse)

In the group of mice infected with the Newcastle strain only 2 mice remained alive 23 hours after the injection of organisms. The dysentery-phage level in the blood of these 2 mice did not show any increase in titer when compared with the titers of the control mice bled at the same time. This was in marked contrast to the rise in titer in the mice which had been injected with phage-susceptible organisms (the X-S45 strain), as is illustrated in figure 1.

*Experiment 3.* This experiment was a repetition of experiment no. 2 but with slight alterations. Before the experiment was started, 2 white mice were injected with 1 ml of a  $10^{-7}$  dilution of a culture of the X-S45 organism in mucin. The culture in this dilution gave a count of 292 organisms per ml or about 73

M.L.D. The mice were bled from the clipped tail at intervals of 15, 30, 60, 90, and 180 minutes after inoculation. A drop of blood from each was diluted in 0.5 ml of broth and plated on MacConkey's agar. The organisms were shown to be present in the blood stream as early as 1 hour after inoculation.

In order to find out how long it took for the dysentery-phage to increase in the blood after the injection of organisms, the intervals of time were altered in this experiment. The mice receiving dysentery-phage alone were bled 3, 12, 24, 72, 96, 120, 144, and 168 hours after the injection of the dysentery-phage. The group of mice which received organisms 96 hours after the injection of the dysentery-phage were bled 3, 12, 24, 48, and 72 hours after injecting the organisms. Thus, there was a parallel series from the 3- to the 72-hour intervals.

Log of the number of  
phage particles per  
ml of blood

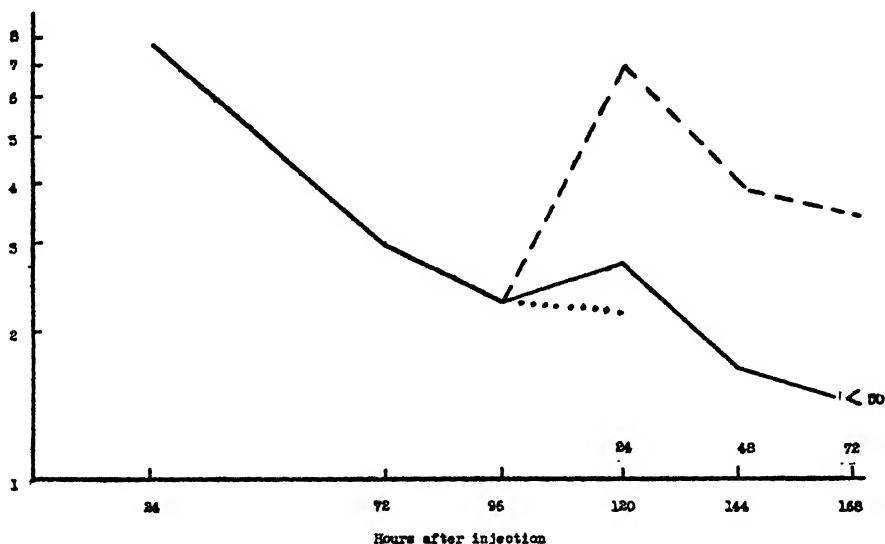


FIG. 1. THE AVERAGE NUMBER OF DYSENTERY-PHAGE PARTICLES PER ML OF BLOOD IN WHITE MICE AS GIVEN IN TABLE 2

———— normal mice; — — — mice injected after 96 hours with phage-susceptible dysentery bacilli, X-S45 strain; . . . . mice injected after 96 hours with phage-resistant dysentery bacilli, Newcastle strain.

The mice inoculated with the homologous organism (X-S45) received only 68 M.L.D. in this experiment, which might serve as an explanation for the proportionally smaller rise in the blood level of bacteriophage in this experiment as compared with that in experiment no. 2.

The mice inoculated with the Newcastle strain (which was not susceptible *in vitro* to the bacteriophage used) received an estimated 166 M.L.D. of the organism. The titer of the bacteriophage was  $4.8 \times 10^9$  particles per ml.

Organisms recovered from the blood of the mice which received both the injection of the X-S45 organism and the homologous bacteriophage, employing the technique of Kligler, Oleinik, and Czazkes (1943), gave the biochemical reac-

TABLE 3

*Blood level of bacteriophage in control mice, in mice infected with phage-susceptible dysentery bacilli (X-S45) and in mice infected with dysentery bacilli not susceptible to the lytic action of the dysentery-phage (Newcastle strain)*

BLEEDING TIME AFTER INJECTION OF DYSENTERY-PHAGE	NUMBER OF DYSENTERY-PHAGE PARTICLES PER ML OF BLOOD IN MICE INJECTED WITH DYSENTERY-PHAGE ONLY	NUMBER OF DYSENTERY-PHAGE PARTICLES PER ML OF BLOOD IN MICE INJECTED WITH X-S45 ORGANISMS, 96 HOURS AFTER INJECTION OF PHAGE	NUMBER OF DYSENTERY-PHAGE PARTICLES PER ML OF BLOOD IN MICE INJECTED WITH NEWCASTLE ORGANISMS, 96 HOURS AFTER INJECTION OF PHAGE
hr			
3	120,000,000		
12	80,000,000		
24	17,000,000		
72	11,000		
96	560		
99	not tested	no plaques seen	not tested
108	not tested	9,800	150 (1 mouse)
120	460	130,000	1,000 (1 mouse)
			<200 (2 mice)
144	200	1,300	
168	60	300	

Log of the number of phage particles per ml of blood

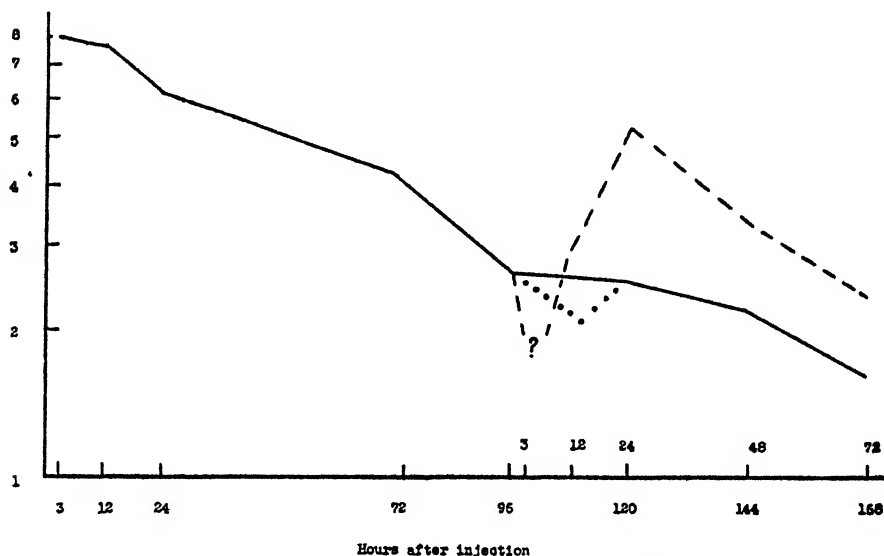


FIG. 2. THE AVERAGE NUMBER OF DYSENTERY-PHAGE PARTICLES PER ML OF BLOOD IN WHITE MICE AS GIVEN IN TABLE 3

———— normal mice; — — — mice injected after 96 hours with phage-susceptible dysentery bacilli, X-S45 strain; . . . . mice injected after 96 hours with phage-resistant dysentery bacilli, Newcastle strain.

tions of the Flexner group of dysentery bacilli and were identical with the stock culture of X-S45 serologically. They also were lysed by the same filtrate used

for injecting the mice. The results of this experiment are shown in table 3 and in figure 2.

Feces from phage-infected mice were found to contain dysentery-phage, whereas feces from mice not infected with dysentery-phage did not contain a bacteriophage active against the strain of dysentery bacilli employed in the experiment.

#### SUMMARY

When a dysentery-phage, prepared against *Shigella paradysenteriae*, Flexner X-S45, is injected intraperitoneally into normal white Swiss mice, it rapidly enters the circulating blood.

The concentration of the dysentery-phage in the blood stream remains high for about 24 hours; however, it diminishes constantly, more or less rapidly at first then rather slowly, until it is barely detectable after 5 to 7 days. It is not eliminated as rapidly as Krueger and Scribner (1941) lead one to believe in their review.

Lytic action *in vitro* is accompanied by protective action *in vivo*; no lytic action *in vitro*, no protective action *in vivo*.

The injection into mice of dysentery bacilli susceptible to the action of the dysentery-phage, after the level of the dysentery-phage in the blood stream is fairly low, causes a temporary (3-hour) decrease in the amount of dysentery-phage in the circulating blood. This decrease is followed by a rapid rise in the amount of dysentery-phage in the circulating blood until a maximum titer is reached in about 12 hours after the injection of the organisms. The rise appears to be roughly proportional to the number of susceptible organisms injected (80,000-fold increase following the injection of 127 M.L.D.). After the maximum amount of dysentery-phage is reached in the blood stream, the titer decreases rapidly within the next 24 hours, thereafter more slowly.

The temporary decrease in the amount of dysentery-phage in the circulating blood, within 3 hours after the injection of phage-susceptible organisms, suggests a concentration of the dysentery-phage at the site of the large number of organisms injected into the peritoneal cavity. The reappearance of dysentery-phage in the circulating blood may come about through one or both of two possible ways. The dysentery-phage liberated in the peritoneal cavity through the lysis of the dysentery bacilli which were injected may enter the blood stream, as happens after dysentery-phage is injected into the peritoneal cavity of normal mice. Following the intraperitoneal injection of virulent dysentery bacilli into normal mice or phage-infected mice, the dysentery bacilli gain entrance to the blood stream. The bacilli are first detected in small numbers 1 hour after injection and are present in greater numbers after 3 hours. Another possible explanation for the increase in the amount of dysentery-phage in the circulating blood between the third and twelfth hours after the intraperitoneal injection of phage-susceptible dysentery bacilli is that those bacilli in the circulating blood undergo lysis in the blood stream. It is reasonable to expect that if the mice are protected against several lethal doses of dysentery bacilli by dysentery-phage, the



bacilli are destroyed wherever phage particles are able to combine with the bacilli. Three hours after the injection of dysentery bacilli into phage-infected mice, both dysentery-phage particles and dysentery bacilli are in the circulating blood.

Regardless of where bacteriophagy takes place *in vivo*, the amount of dysentery-phage in the circulating blood increases during the course of infection with phage-susceptible dysentery bacilli.

Dysentery-phage is an unique antibiotic in that as it protects animals from fatal infections with dysentery bacilli more dysentery-phage is produced.

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# THE PROTECTIVE ACTION OF DYSENTERY BACTERIOPHAGE IN EXPERIMENTAL INFECTIONS IN MICE

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A review of the literature on the *in vivo* action of dysentery-phage does not enable one to formulate any definite conclusions as to its value (Morton and Engley, 1945). There are about twice as many favorable as unfavorable reports. However, the use of dysentery-phage in man has been made under such poorly planned or executed experiments as to invalidate the results. The logical sequence of events should have been the testing of dysentery-phage in experimental animals, after the discovery of its action *in vitro*, before trying it on humans. To our knowledge this was not done. We wished to know whether dysentery-phage had any action *in vivo* and whether it would be reasonable to expect any prophylactic or therapeutic action in man. We feel that the results of animal experiments to be reported answer both questions in the affirmative. The tests were first conducted in November, 1942, under conditions which required secrecy. They were subsequently repeated under conditions which warrant publication.

During the studies we were further encouraged by the following reports: Rakietsen and Rakietsen (1943) demonstrated protective action of Flexner-phage in developing chick embryos. MacNeal, Blevins, and Pacis (1943) proved protective action of Sonne-phage, likewise, in developing chick embryos. Dubos, Straus, and Pierce (1943) showed protective action of Shiga-phage in mice. Our results (Morton and Engley, 1944) with Flexner-phage in mice substantiate the findings of the above workers and in addition point out some quantitative relationship of bacteriophage to microorganisms *in vivo*. These relationships we feel are very important in understanding bacteriophagy *in vivo* and in attempting to project the results obtained in one species of experimental animal to other species of animals, including man.

## MATERIALS

**Bacteriophage.** Dysentery-Y phage, originally obtained from Professor Müller, Hygien-Institute, Köln-Lindenthal, by Prof. Stuart Mudd in 1932 and used repeatedly by the senior author in the Department of Bacteriology since that time, was employed. It was always generated against Flexner-Y, strain P107. This preparation has been observed to retain its activity over four and one-half years of storage in the dark at room temperature.

Dysentery-XS45 phage was produced by adapting the dysentery-Y phage to the X-S45 strain by the present authors. The dilution factor was such that it was impossible for any of the original Y-phage to be present in the filtrate used as XS45-phage.

*Test organisms.* *Shigella paradysenteriae*, Flexner X-S45, obtained in lyophilized form from Miss Beatrice Doak after several mouse passages. It originally came from the Army Medical School. *Shigella paradysenteriae*, Y, P107. University of Pennsylvania stock culture, originally obtained by Prof. Stuart Mudd from Professor Müller in 1932. *Shigella paradysenteriae*, Z-911A, obtained in lyophilized form from Miss Beatrice Doak after several mouse passages. It originally came from the Porto Rico Department Laboratory, U. S. Army, P. R. *Shigella paradysenteriae*, no. 88, Boyd strain M-265, obtained in lyophilized form from Miss Beatrice Doak after mouse passages. It was originally obtained from the Royal Army Medical College.

*Culture media.* For growing the dysentery strains preparatory to phage lysis the following tryptone glucose yeast-extract medium was employed:

Tryptone peptone (Bacto).....	20 g
Glucose (cerelose).....	5 g
NaCl.....	5 g
Yeast extract (Bacto).....	2 g
Dipotassium phosphate ( $K_2HPO_4$ ).....	2.5 g
Distilled water.....	1000 ml
Final pH 7.5	

For plate counts of bacteriophage plaques and bacterial counts the following extract agar was used:

Beef extract (Bacto).....	3 g
Parke-Davis peptone.....	10 g
NaCl.....	5 g
Agar (granular).....	20 g
Distilled water.....	1000 ml
Final pH 7.5	

*Test animals.* White Swiss mice weighing 17 to 20 grams each, which were obtained from Rockland Farms, were used for all experiments herein reported.

*Filters.* All filtrations were carried out with pyrex brand UF fritted glass filters (Morton, 1943, 1944).

*Mucin.* A suspension of 5 per cent gastric mucin prepared according to Miller (1935) was used for suspending the infecting organisms before intraperitoneal injection. We are indebted to Dr. Seymour P. Halbert for the supply of mucin.

#### TECHNIQUE

A tryptone glucose yeast-extract medium was utilized in all preparations of dysentery bacteriophage in these experiments. Such an enriched medium allowed maximum bacterial growth in a minimum length of time after inoculation. This removed the possibility that a large amount of toxic end products might be formed before use and gave the phage a large number of young susceptible cells for lysis.

The phage was generated in 250-ml centrifuge bottles containing 150 ml of medium. These were inoculated with 2 ml of an overnight broth culture of the

susceptible organism and incubated in a 37 C water bath. Each bottle was fitted with a two-hole rubber stopper, and sterile filtered air was allowed to bubble slowly through the medium. After 2½ to 3 hours a fairly heavy growth resulted, and 1 ml of a stock phage was added to the susceptible cells. Incubation under the same conditions was continued for 3 hours, and the lysate was filtered through pyrex brand UF fritted glass filters. Each lot of lysate was checked by plating out 0.2 ml of serial dilutions made in standard amounts of young cultures. Plaque counts from the plates were taken as being equivalent to the number of bacteriophage particles in the lysate. The titers ranged between  $5.5 \times 10^9$  and  $2 \times 10^{11}$  with the most frequent titer about  $1 \times 10^{10}$ . The filtrates were stored in sterile screw-cap bottles at 4 to 11 C until used. The titers of the phages were rechecked when used.

The bacterial cultures used for infecting agents were grown in the tryptone glucose yeast-extract medium. A lyophilized virulent culture was planted in broth and incubated overnight. On the following morning 0.5 ml was incubated in 25 ml of broth for 5 hours in the 37 C water bath with air bubbling through the medium. This culture was centrifuged, and the supernatant replaced with an equivalent amount of broth. This preparation contained approximately  $4 \times 10^9$  organisms per ml. Serial dilutions were made in broth for (a) accurate plate counts and (b) mucin suspensions for mouse injections. All injections of mucin suspensions were given in 1-ml amounts intraperitoneally with 22-gauge needles.

Control mice received an amount of tryptone glucose yeast-extract broth equal to the volume of dysentery-phage injected. In the prophylaxis experiment the control mice received an inactivated dysentery-phage. The dysentery-phage was inactivated by heating at 65 C for 60 minutes. It showed no lytic activity when tested against susceptible organisms *in vitro*.

#### EXPERIMENTAL

*What is the minimum amount of dysentery-phage which will protect mice when administered simultaneously with the infecting dose of dysentery bacilli?*

The proper dilution of culture was mixed with 5 per cent sterile gastric mucin suspension in the ratio of 0.1 ml of culture and 0.9 ml of mucin. One ml of the suspension was injected intraperitoneally into white mice weighing 17 to 20 grams. Within a minute 1 ml of the diluted dysentery-phage was also injected intraperitoneally. All deaths occurred within 80 hours. The results of the experiments are given in table 1.

*Conclusions.* Strain X-S45 of *Shigella paradysenteriae*, Flexner, is lysed *in vitro* by its homologous bacteriophage, X-S45. Administered intraperitoneally into white mice immediately after the infecting dose of organisms, the dysentery-phage protects mice when the ratio of phage particles to bacterial cells is approximately 1:8.

Strain Z-911A of *Shigella paradysenteriae*, Flexner, is lysed *in vitro* by dysentery-phage produced against strain X-S45. The dysentery-phage is also active against the organisms *in vivo*, protecting white mice when the ratio of phage particles to bacterial cells is approximately 1:7.

Strain X-S45 of *Shigella paradysenteriae*, Flexner, is lysed *in vitro* by dysentery-phage produced against a Y strain. The dysentery-phage is also active against the organisms *in vivo*, protecting white mice when the ratio of phage particles to bacterial cells is approximately 1:5.

TABLE 1

*Minimum amount of dysentery-phage required to protect white mice when injected intraperitoneally immediately following the infective dose of dysentery bacilli*

CULTURE			DYSENTERY-PHAGE			MICE		
Strain	No. of bacteria*	No. of lethal doses†	Strain	Dilution	No. of phage particles‡	No. injected	No. surviving	No. dying
X-S45§.....	40,000	10,000	X-S45	$1 \times 10^{-4}$	5,000,000	3	3	0
X-S45.....	40,000	10,000	X-S45	$1 \times 10^{-5}$	500,000	3	3	0
X-S45.....	40,000	10,000	X-S45	$1 \times 10^{-6}$	50,000	3	3	0
X-S45.....	40,000	10,000	X-S45	$1 \times 10^{-7}$	5,000	3	2	1
X-S45.....	40,000	10,000	X-S45	$1 \times 10^{-8}$	500	3	0	3
Z-911A§.....	34,000	11,300	X-S45	$1 \times 10^{-6}$	50,000	3	3	0
Z-911A.....	34,000	11,300	X-S45	$1 \times 10^{-7}$	5,000	3	3	0
Z-911A.....	34,000	11,300	X-S45	$1 \times 10^{-8}$	500	3	0	3
X-S45  .....	48,000	12,000	Y	$1 \times 10^{-4}$	1,000,000	3	3	0
X-S45.....	48,000	12,000	Y	$1 \times 10^{-5}$	100,000	3	3	0
X-S45.....	48,000	12,000	Y	$1 \times 10^{-6}$	10,000	3	2	1
X-S45.....	48,000	12,000	Y	$1 \times 10^{-7}$	1,000	3	0	3
Newcastle 88¶.....	30,000	10,000	X-S45	undil.	50 billion	3	0	3
Newcastle 88.....	30,000	10,000	X-S45	$1 \times 10^{-1}$	5 billion	3	0	3
Newcastle 88.....	30,000	10,000	X-S45	$1 \times 10^{-2}$	500 million	3	0	3

\* Determined by preparing poured plates from serial dilutions and counting the number of colonies which developed upon incubation.

† Estimated after determining the minimum number of organisms required to kill 2 out of 3 mice.

‡ Estimated by making serial dilutions of the dysentery-phage in young broth cultures of the homologous strain, depositing a measured amount on the surface of a sterile agar plate, spreading uniformly over the surface with a glass spreader, and counting the number of plaques which develop upon incubation of the plates.

§ Lysed *in vitro* by X-S45 dysentery-phage.

|| Lysed *in vitro* by Y dysentery-phage.

¶ Not lysed *in vitro* by X-S45 dysentery-phage.

The Newcastle-88 strain of *Shigella paradysenteriae* is not lysed *in vitro* by our strain of dysentery-phage produced against strain X-S45, and it appeared to have no protective action *in vivo*. This dysentery-phage does not protect white mice when the ratio of phage particles to bacterial cells is approximately 1,700,000 to 1.

*Discussion.* The results of the experiment indicate (1) that a strain of dysentery bacilli lysed *in vitro* by dysentery-phage is also destroyed *in vivo* and (2) that less than one bacteriophage particle per bacterium gives protection *in vivo*.

This is also substantiated by Fisk (1938), who found a ratio of approximately 1 phage particle to 30 typhoid organisms would give protection *in vivo*. Ward (1943), working with Vi typhoid bacteriophage, found that a ratio of 1 phage particle to 2 organisms would protect 91 per cent of the mice injected, whereas a ratio of 1 phage particle to 23 organisms protected 40 per cent of the mice. (These facts were not reported as such by these authors but were deduced by analysis of their results.)

Rakieta and Rakieta (1943), investigating experimental *Shigella paradysenteriae*, Flexner, infection in developing chick embryos, showed a definite increase in the bacteriophage titer after adding measured amounts of dysentery-phage. Dubos *et al.* (1943), working with intracerebral *Shigella dysenteriae* infections in mice, reported an increase in dysentery-phage in both the brain substance and blood stream after parenteral injection of specific bacteriophage. Morton and Perez-Otero (1944) have proved that dysentery-phage injected intraperitoneally enters the blood stream and disappears over a period of a week. When phage-susceptible dysentery bacilli are injected intraperitoneally at a time when the dysentery-phage titer is low, there is a rapid and significant rise in the titer of the dysentery-phage in the circulating blood. The results of other investigators as well as the results of experiments reported in this paper prove that, under appropriate conditions, dysentery-phage increases *in vivo*.

*How long after the intraperitoneal injection of an infecting dose of dysentery bacilli may dysentery-phage be withheld and still prevent death of white mice?*

The answer to this question is important in determining the therapeutic value of dysentery-phage against infection with the dysentery bacillus. The infecting dose consisted of 49,000 dysentery bacilli of the X-S45 strain, or approximately 12,000 M.L.D., suspended in 5 per cent sterile gastric mucin, and injected intraperitoneally into white Swiss mice which averaged about 17 grams each. The dysentery-phage was generated against the X-S45 strain and had a titer of  $1 \times 10^{11}$  particles per ml.

*Conclusions.* One-ml amounts of dysentery-phage (containing  $1 \times 10^{11}$  phage particles per ml) generated against a virulent strain of *Shigella paradysenteriae*, Flexner X-S45 strain, are not toxic for white mice when injected intraperitoneally.

The dysentery-phage may not be withheld many hours after the administration of many minimum lethal doses (approximately 12,000) of dysentery bacilli. The dysentery-phage failed to protect 2 out of 3 mice when it was withheld 6 hours. Typical dysentery bacilli were isolated from the heart blood of several of the mice which died during the course of the experiment.

*Discussion.* The results (table 2) indicate that in an infection coming to a rapid termination, such as experimental paradysentery infections in mice, bacteriophage administration cannot be delayed very long after infection. Fisk (1938) allowed a period of 4 hours to elapse before treatment with typhoid-phage in experimental typhoid infections and obtained successful results. Ward (1943) obtained similar results in the same type of infection, even when the typhoid-phage was given intravenously. Asheshov, Wilson, and Topley (1937) found these to be true irrespective of mode of injection. When these authors withheld

bacteriophage longer than 4 hours after the infecting dose, unsuccessful results were obtained. In clinical infections emphasis has been placed on early treatment with bacteriophage in order to obtain the best results.

*How effective as a prophylactic agent is dysentery-phage when given at various intervals of time prior to the infective dose of virulent dysentery bacilli?*

TABLE 2

*The effect of withholding the doses of dysentery-phage for varying periods of time after the infecting dose of dysentery bacilli*

NO. OF MICE	NO. OF BACILLI INJECTED	NO. OF M.L.D.	AMOUNT OF DYSENTERY-PHAGE INJECTED	HOURS ELAPSED AFTER INFECTING DOSE BEFORE ADMINISTRATION OF PHAGE	RESULT
3	0 (controls)	0	1 ml	0	All survived
2	48,000	12,000	0 (controls)	0	1 dead in 31 hours* 1 dead in 31 hours
3	48,000	12,000	1 ml	0	All survived
3	48,000	12,000	1 ml	3	1 dead in 31 hours† 2 survived
3	48,000	12,000	1 ml	6	1 dead in 31 hours* 1 dead in 43 hours 1 survived
3	48,000	12,000	1 ml	9	1 dead in 22 hours* 2 dead in 43 hours
3	48,000	12,000	1 ml	12	1 dead in 22 hours* 2 dead in 43 hours
3	48,000	12,000	1 ml	15	1 dead in 26 hours* 1 dead in 80 hours 1 survived
3	48,000	12,000	1 ml	18	1 dead in 22 hours* 1 dead in 43 hours 1 dead in 80 hours

\* Heart blood cultured after death and dysentery bacilli isolated.

† Heart blood cultured after death but no dysentery bacilli isolated.

Dysentery-phage, as the filtrate of a culture of dysentery bacilli which has undergone lysis, contains, in addition to the specific dysentery-phage, the metabolic products of the dysentery bacilli and the soluble cellular products of lysis. These soluble cellular components may serve as antigens and produce an active immunity in the animal body. Since antibodies do not appear in appreciable amounts in the circulation of an otherwise normal animal until about the seventh

to the tenth day, 7 days were taken as the longest prophylactic period. Every day for 7 days 3 normal mice were injected intraperitoneally with 1 ml of dysentery-phage of a titer of  $1 \times 10^9$  particles per ml. As a control, for ruling out the effect of active immunization, an equal amount of the same dysentery-phage, which had been heated at 65 C for 60 minutes, was injected into an equal number

TABLE 3

*The effectiveness of an intraperitoneal injection of dysentery-phage given at various periods of time before the injection of several lethal doses of dysentery bacilli*

TIME ELAPSING BETWEEN THE PROPHYLACTIC DOSE OF PHAGE AND THE INFECTIVE DOSE OF DYSENTERY BACILLI	NUMBER OF MICE IN- JECTED WITH DYSENTERY- PHAGE	NUMBER OF VIRULENT DYSENTERY BACILLI INJECTED	RESULT	NUMBER OF MICE INJECTED WITH HEAT- INACTIVATED DYSENTERY- PHAGE	NUMBER OF VIRULENT DYSENTERY BACILLI INJECTED	RESULT
<i>days</i>						
7	3	40,000	1 died on 5th day 2 survived	3	40,000	1 died at 19 hr 1 died on 5th day 1 survived
6	3	40,000	All survived	3	40,000	1 survived 1 died in 20 hr 1 died in 41 hr
5	3	40,000	All survived	3	40,000	1 survived 1 died in 20 hr 1 died in 24 hr
4	3	40,000	2 survived 1 died in 19 hr	2	40,000	2 died in 19 hr
3	3	40,000	All survived	3	40,000	All died in 19 hr
2	3	40,000	2 survived 1 died in 19 hr	3	40,000	1 died in 19 hr 1 died in 24 hr 1 died in 29 hr
1	3	40,000	All survived	3	40,000	1 died in 19 hr 1 died in 29 hr 1 survived
0	3	40,000	2 survived 1 died in 22 hr	3	40,000	3 died in 19 hr

of normal mice under the same conditions. The heat-inactivated dysentery-phage showed no lytic action against the culture when tested *in vitro*. The culture used was *Shigella paradysenteriae*, Flexner X-S45. The challenging dose was about 40,000 organisms, or 10,000 minimum lethal doses of dysentery bacilli. The test animals were white Swiss mice which weighed 17 to 20 grams each.



*Conclusions.* The injection of heat-inactivated dysentery-phage appears to produce some immunity in mice after 5 to 7 days.

Heat-inactivated dysentery-phage was without effect in protecting mice against 10,000 minimum lethal doses of dysentery bacilli when given simultaneously or as long as 4 days prior to the injection of virulent dysentery bacilli.

Dysentery-phage was effective in protecting at least two of three mice against injections of at least 10,000 minimum lethal doses of the homologous strain of dysentery bacilli when given simultaneously or as long as 7 days prior to the injections of virulent dysentery bacilli.

*Discussion.* Krueger and Scribner (1941) have claimed that much of the protection resulting from bacteriophage preparations comes from immune responses. Active immunity does not develop to any great extent for at least 4 or 5 days; Ehrlich (quoted by Boyd, 1943) found that in mice 6 days were necessary for antibody formation. Boyd states that after one injection of antigen several days elapse before any appearance of antibody is noted. The maximum response is found at 7 days; thereafter a drop in titer occurs. In our experiments most deaths in the experimental animals occurred within 48 hours after infection, thus leaving little possibility of immune reactions being responsible for the protective action of dysentery-phage. Gay and Chickering (1916) suggested that the protein shock reaction is responsible for the protective action of such protein preparations. In our experiments we used both culture media proteins and heat-inactivated dysentery-phage (65 C for 60 minutes) as controls. Neither of these substances gave protection when given at the same time as the infecting dose. The inactivated dysentery-phage preparation used in the prophylaxis experiments failed to give any protection when given as long as 4 days before infection. Some protection was noted, however, after 4 days when 1 of 3 test mice in each group survived 10,000 M.L.D.'s. This protection did not compare with that provided by the active dysentery-phage preparation.

*Can one strain of dysentery-phage protect against more than one Shigella species?*

Working with the dysentery-phage developed against P107, a Y-dysentery strain, and the same strain of phage generated against a different strain of dysentery organism, namely X-S45, an attempt was made to find out the number of dysentery cultures each would lyse *in vitro*.

*Technique.* Each dysentery strain to be tested was grown overnight in broth, inoculated into three test tubes containing 5 ml of tryptone glucose yeast-extract broth and incubated in a 37 C water bath until slight turbidity was noticeable (2½ to 3 hours). The 0.5 ml of X-S45 phage was added to one tube, 0.5 ml of Y-phage was added to the second tube, and the third tube was used for a control on the amount of growth. The tubes were shaken thoroughly, and then 0.2 ml of culture from each tube was placed on a separate agar plate with a separate pipette and spread evenly over the entire surface with a separate sterile glass spreader. The plates were incubated at 37 C for 18 to 24 hours. Lytic activity and growth could be noted, however, in 6 to 8 hours on the plates. Lysis of a culture was noted either by the complete absence of growth or the presence of

plaques, whereas the control plate showed a uniform film of growth. Quantitative lysis was noted by the + system, but the results will be reported here as qualitative, i.e., presence or absence of lysis.

*Discussion.* If the Alkalescens, Boyd, Newcastle, and Schmitz varieties, which are not attacked by our two strains of dysentery-phages, are omitted from the final considerations, the percentage of cultures in those varieties of dysentery bacilli definitely attacked by the two dysentery-phages becomes 68 per cent.

Of the 460 strains of *Shigella paradysenteriae* tested with dysentery-phage against X and Y strains only 2 more Flexner strains were attacked by the dysentery X-phage than by the Y-phage, whereas 2 more Sonne strains were attacked by the Y-phage than by the X-phage. There were also quantitative differences in the susceptibility of various strains to lysis by the two dysentery-phages.

TABLE 4

*Susceptibility of various strains of Shigella to the action of dysentery-X and Y phages*

CULTURES	STRAINS LYSED BY X-PHAGE	STRAINS LYSED BY Y-PHAGE	STRAINS NOT LYSED BY X-PHAGE	STRAINS NOT LYSED BY Y-PHAGE	TOTAL NUMBER OF CULTURES TESTED	PER CENT OF STRAINS LYSED BY X-PHAGE	PER CENT OF STRAINS LYSED BY Y-PHAGE
Shiga.....	4	4	8	8	12	33	33
Flexner.....	153	151	60	62	213	71.3	70.8
Boyd strains.....	1	1	5	5	6		
Schmitz.....			20	20	20		
Sonne.....	38	40	22	20	60	63.3	66
Alkalescens.....	4	4	90	90	94	4.2	4.2
Newcastle....	1	1	29	29	30	3.3	3.3
Strong.....	2	2			2		
Mt. Desert....	2	2	2	2	4		
Dispar.....	14	14	2	2	16	87.7	87.7
Hiss-Y.....	2	2	1	1	3		
Totals .. ..	221	221	239	239	460	48	48

These two preparations of dysentery-phages seem to have a fairly broad reactivity. With the Flexner variety 71 per cent of 213 strains were lysed. Over 60 per cent of the 60 Sonne strains were lysed. A varying number of strains of the other varieties of *S. paradysenteriae* were also lysed. Since the two races of dysentery-phages were developed against Flexner organisms, it might be expected that the greatest number of these cultures would be susceptible to lysis by these particular dysentery-phages.

These strains of the *Shigella* genus were obtained from many sources.<sup>1</sup> They were isolated by workers in Massachusetts, Florida, Mississippi, Virginia, West Virginia, New Mexico, New York, and other states, as well as from Puerto Rico, Cuba, Philippine Islands, Germany, Canada, England, India, Italy, Nigeria, and

<sup>1</sup> We are indebted to Dr. Joseph Felson, Bronx Hospital, New York, who graciously placed at our disposal the strains of *Shigella* on deposit in the Dysentery Registry.

South America. The authenticity of the cultures was not checked because they were received, for the most part, from experienced workers in the field of dysentery research and were employed merely as a preliminary qualitative survey.

#### SUMMARY

Dysentery-phage shows good protection, prophylactically and therapeutically, against experimental infections in mice produced by *Shigella paradysenteriae*, Flexner.

Lysis *in vitro* of dysentery bacilli by dysentery-phage is an indication of activity *in vivo*; no activity *in vitro*, no activity *in vivo*.

Quantitative studies show that dysentery-phage can protect mice in the ratio of 1 phage particle to 8 dysentery bacilli, when doses of 10,000 minimum lethal doses of organisms are employed.

Treatment with dysentery-phage may be delayed for 3 hours after the infecting dose of organisms is given. If the dysentery-phage is given 6 hours following the infection, little protection is afforded.

Dysentery-phage has been observed to exert a prophylactic action when given as long as 7 days preceding the infecting dose of 10,000 minimum lethal doses of dysentery bacilli.

Heat-inactivated dysentery-phage showed no prophylactic action when given as long as 5 days preceding the infecting dose of 10,000 minimum lethal doses of dysentery bacilli.

A strain of dysentery-phage has been studied which has a fairly broad reactivity within the genus *Shigella*.

Adaptation of this strain of dysentery-phage to a different strain of dysentery bacilli had little effect on its range of reactivity.

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# THE RELATION OF SPORULATION AND THE RANGE OF VARIATION OF THE HAPLOPHASE TO POPULATIONAL ADAPTATION<sup>1</sup>

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## INTRODUCTION

In previous publications (Spiegelman, Lindegren, and Hedgecock, 1944; Spiegelman and Lindegren, 1944) the authors have shown that in certain haploid strains populational adaptation to galactose fermentation may involve mutation and subsequent selection of the adaptable type. Using two diploid strains of *Saccharomyces cerevisiae* they established that in other strains adaptation could be effected by a direct interaction between the substrate and the cytoplasm of the existent cells. These experiments emphasized the important point that the particular biological mechanism involved in the production of a given enzyme or enzyme system in a population of cells is a characteristic of the strain being examined rather than of the enzyme system itself. It became apparent that to solve the problem of the biological mechanisms involved in any particular case of adaptation reference must be made to the genetic background and stability of the population being studied.

These same experiments presented the opportunity of analyzing the problem of unadaptability. From the earliest investigations by Armstrong (1905) and Kluver (1914) the existence of unadaptable yeasts was noted. Subsequent investigations have uncovered many more. As may be seen from a perusal of Stelling-Dekker's monograph on the sporogenous yeasts (1931), examples of nonfermenters of galactose exist in all the genera. As a matter of fact, Stelling-Dekker has used fermentability with respect to galactose and several other sugars as the basis of identification within several of the yeast genera. An examination of the mechanisms whereby nonfermenting types gain or lose this character is of some importance in evaluating the phylogenetic significance of these properties.

The question has in addition taken on some practical significance because of the tendency in recent years to use various yeasts as tools in the analysis of sugar mixtures. Recently, for example, Wise and Appling (1944) published a method for determining galactose in mixtures with other hexoses. They used *Saccharomyces carlsbergensis*, which could ferment galactose, in conjunction with *Saccharomyces bayanus*, which could not. They also reported that certain galactose-fermenting strains of *S. cerevisiae* had apparently lost that ability, at least partially, within a 5-year period. Our earlier experiments suggest that

<sup>1</sup> This investigation was aided by a grant from the Penrose Fund of the American Philosophical Society.

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the retention by a nonfermenter of this character after a long period of contact with galactose might be due to its genetic stability. Thus, a nonsporulating population of diploids, or one in which some other mechanism existed for the suppression of the haplophase, would be unlikely to gain a new character or lose an old one by mutation. This suggested the possibility of attempting to adapt strains which had been previously labeled as nonfermenters by encouraging the production of haploids and thus disturbing the genetic stability of the population. The same type of experiments could also be used to investigate the conditions for the reverse phenomenon, that is, the loss of adaptability by adaptable strains. The basis for the method of inducing haploidy within a diploid population consisted essentially in stimulating sporulation, the details of which are described in the section on methods.

It is the purpose of the present paper to describe the experiments which lead to the adaptation to galactose fermentation of a previously unadaptable form. This will be compared with the negative results obtained with the same methods on other forms, in which additional mechanisms for suppression of the haplophase exist, which tended to minimize the effect of heavy sporulation. Experiments will also be discussed in which unadaptable progenies were isolated from adaptable parents. The latter experiments indicate that the range of biochemical variation of mutant types may become limited as compared with the parent types from which they arose.

#### MATERIALS AND METHODS

*Strains of yeast.* Three yeast species, *Schizosaccharomyces pombe*, *Schizosaccharomyces octosporus*, and *Saccharomyces ludwigii*,<sup>3</sup> were chosen among the known nonfermenters of galactose. These particular ones were selected for this study for several reasons. All three were included among the yeasts examined by Armstrong (1905). He concluded from his study that they were incapable of adaptation to galactose fermentation. In addition to the fact that they have been studied more thoroughly than other nonfermenters of galactose, they have another advantage of some importance from a comparative point of view: *S. pombe* can without any difficulty exist in the haplophase; this is not true of *S. octosporus*, and still less so for *Saccharomyces ludwigii*.

Although *S. octosporus* sporulates with ease, the haploids which result from the germination fuse rapidly to produce diploid cells. When four- and eight-spored asci from the stock culture were dissected and the spores planted, they all grew, and every single-ascospore culture sporulated on the agar in less than 48 hours. The further spore analysis of *S. octosporus* indicates that the diploid stock culture was completely homozygous and that, unlike *Saccharomyces cerevisiae*, the production of viable spores apparently does not depend on the pre-existence of a heterozygous nucleus. The sporulation phenomenon of a

<sup>3</sup> These as well as *Saccharomyces carlsbergensis* were obtained from the yeast collection of the Northern Regional Research Laboratory at Peoria, Illinois, through the courtesy of Dr. L. J. Wickerham.

single-spore culture is not observed in the *S. cerevisiae* strains employed. The fact that it does occur in *S. octosporus* is an indication of heavy diploidization, confirming the direct microscopic observations (see fig. 1, discussed below). This process of immediate fusion effectively suppresses the haplophase in any culture in which it occurs.

This suppression mechanism is even more highly developed in the case of *Saccharomyces ludwigi*. Guilliermond (1903) reported that this yeast usually



FIG. 1. FIELD OF A SPORULATING CULTURE OF SCHIZOSACCHAROMYCES OCTOSPORUS (SEE TEXT FOR DESCRIPTION)

forms four spores without previous conjugation. On germination, however, the spores conjugate within the mother cell two by two, so that only two vegetative cells emerge from each 4-spored ascus. Winge and Laustsen (1939) confirmed these observations and described the successful isolation of the haplophase by a micromanipulative dissection of the ascospore and separation of the four spores before germination. As a result of their examination of the haplophase cultures, they concluded that *Saccharomyces ludwigi* was a bal-



anced heterozygote. The net result of the germination mechanism is the almost complete suppression of the haplophase under normal conditions.

The adaptable strains from which unadaptable progenies were isolated consisted of two *Saccharomyces cerevisiae* strains (Db23B and Lk2(12) previously studied in this laboratory (Spiegelman, Lindegren, and Hedgecock, 1944; Spiegelman and Lindegren, 1944) and *S. carlsbergensis* which was tested and found to be rapidly (90 minutes) adaptable to galactose fermentation.

*Media.* One liter of the basic medium contained the following substances: 2 g of autolyzed yeast extract powder, 3 g of bacto-peptone, 1 g of  $(\text{NH}_4)_2\text{SO}_4$ , 2 g of  $\text{KH}_2\text{PO}_4$ , 0.25 g of  $\text{MgSO}_4$ , 0.13 g of  $\text{CaCl}_2$ , 1 ml of 50 per cent sodium lactate. To this was added the desired carbohydrate. The medium used for adaptation of mixed haploid and diploid populations contained, in addition to the above, 80 g of galactose and 20 g of glucose.

*Carbohydrates.* Reagent grade glucose and galactose were used in the media without further purification. The galactose used in the manometric test for adaptation (see below) was further purified according to a method modified from that used by Stephenson and Yudkin (1936). The final solution obtained with their method was evaporated under reduced pressure and the galactose was recrystallized from 70 per cent alcohol. The crystals were then redissolved and again recrystallized, and dried in vacuo at 60 C°.

*Tests for adaptation.* All cultures were grown at 29 C°. A sample of the culture to be tested was centrifuged from its medium and washed twice in large volumes of M/15  $\text{KH}_2\text{PO}_4$ . The cells were then resuspended in M/15  $\text{KH}_2\text{PO}_4$  and made up to the desired density using a nephelometer. Samples were taken for the determination of dry weights when necessary. A sample (1.8 ml) of this suspension was then placed in a Warburg cup with 0.2 ml of 20 per cent purified galactose in the side arm. After being flushed with nitrogen, the stop-cocks were closed and the galactose tipped in. The nitrogen used to displace the air in measurements of anaerobic  $\text{CO}_2$  production was passed over hot copper to remove any traces of oxygen. All measurements were taken at 30.2 C°, the vessels being shaken at a rate of 100 oscillations per minute over a 7-cm arc. A  $Q_{\text{CO}_2}^N$  of 100 or above with galactose was taken as adaptation. In practice lower values were not found; either the rate of  $\text{CO}_2$  evolution was zero and remained there or it could be made to reach 100 and exceed it by continued culturing in the presence of galactose.

*Sporulation.* All the diploid strains employed in the present study sporulated readily if left standing over 7 days either in a fluid culture medium or on an agar slant. When particularly heavy sporulation was desired, a method involving inoculation on a presporulating medium and seeding suspensions from this on a Graham and Hastings (1941) gypsum block was used (Lindegren and Lindegren, 1944).

*Oxygen consumption and  $\text{CO}_2$  production.* The oxygen consumption ( $Q_{\text{O}_2}$ ) and aerobic  $\text{CO}_2$  production ( $Q_{\text{CO}_2}^{\text{O}}$ ) were measured by the Warburg manometric technique. Forty-eight-hour glucose-grown cultures washed twice in M/15  $\text{KH}_2\text{PO}_4$  were always employed. The aerobic  $\text{CO}_2$  production was measured

by the usual two-cup method. Inaccuracies owing to retention of the  $\text{CO}_2$  were reduced to a minimum by using  $\text{M}/15 \text{ KH}_2\text{PO}_4$  as a suspending medium. All measurements were made at  $30.2^\circ \text{C}$ . The anaerobic  $\text{CO}_2$  production ( $Q_{\text{CO}_2}^{\text{N}}$ ) of unadapted cells was measured by the method already described.

#### EXPERIMENTAL RESULTS

*Adaptation of Schizosaccharomyces pombe to galactose.* Preliminary experiments indicated that the strain of *Schizosaccharomyces pombe* used sporulated readily if allowed to stand over 7 days in the culture medium. If the cultures were transferred every other day, however, no spores were observed.

To test the effect of sporulation on adaptation, several different kinds of experiments were performed. Twelve flasks each containing 60 ml of 8 per cent glucose liquid medium were inoculated and allowed to stand in the incubator for 20 days. At the end of this period, examination of the growth on the bottom of the flasks showed that in two of them 20 per cent or more of cells had sporulated. The supernatant medium was poured off from these two, and each mixture of cells and spores was resuspended in 300 ml of 8 per cent galactose and 2 per cent glucose adaptation medium and placed in the incubator. Twenty-ml samples were removed at two-day intervals, and the cells centrifuged down and washed in  $\text{M}/15 \text{ KH}_2\text{PO}_4$ . After resuspension in the buffer fluid, they were tested for adaptation to galactose fermentation by the manometric method previously described. One of the cultures so prepared adapted itself in 8 days, the other in 6. The 2 per cent glucose included in the galactose medium was found necessary after many unsuccessful attempts without its addition. It serves the function of allowing the spores to germinate and of giving the haploids an opportunity to divide rapidly and accumulate in large numbers. After the exhaustion of the glucose, the galactose which still remains can then select from the heterogeneous population any existent fermenters.

In another experiment the growth in 24-day agar slants containing between 10 and 20 per cent sporulating cells was washed with sterile broth into the glucose galactose medium. Out of four attempts only one finally yielded an adaptable strain. Finally, the growths on gypsum blocks containing well over 30 per cent of sporulating cells were heavily inoculated into the adapting medium. The results and time required for adaptation are given in table 1. All adapted themselves within 6 days.

While these experiments were being performed, two kinds of control series were run. Cultures originating from the same stock strain of *Schizosaccharomyces pombe* were transferred every other day in the adapting medium. As was previously noted, sporulation does not occur under these conditions. Adaptation did not occur over a 3-month period during which these cultures were transferred and observed. In another type of experiment the original culture was inoculated into the basic medium containing 8 per cent of the purified galactose and left standing without transfer. None of the 12 tubes so prepared showed any evidence of adaptation to galactose fermentation at the end of 78 days.

*Experiments with Schizosaccharomyces octosporus and Saccharomycodes ludwigii.* Experiments exactly like those described in the previous section were also carried out with *Schizosaccharomyces octosporus* and *Saccharomycodes ludwigii* but without success. *S. octosporus* is one of the best sporulating yeasts examined in this laboratory, usually up to 80 per cent. Figure 1 is a representative field of a sporulating culture of this strain. This same picture demonstrates why sporulation is not sufficient to disturb the genetic stability of the culture.

In addition to the heavy-walled spores contained in the asci, the majority of the cells seen are the oblong, diploid cells. In the lower left-hand corner may be seen 4 small, round haploids arranged in pairs. The upper pair has already begun the process of elongation to form the conjugation tubes. Another pair in the lower right-hand corner is much further along in the process. Isolated haploid cells are very rarely seen in these cultures. Since under these conditions the haploids do not exist very long as such in the population, any genetic instability they may have cannot express itself in terms of changing

TABLE I  
*Adaptation of Schizosaccharomyces pombe to galactose fermentation*

EXPERIMENT	ORIGIN OF HEAVILY SPORULATING CULTURES (20% AND ABOVE)	PERIOD IN DAYS REQUIRED FOR APPEARANCE OF ADAPTATION
1	20-day broth culture	8
2	"	6
3	24-day agar slant	12
4	Gypsum block	2
5	"	6
6	"	4
7	"	4

populational characteristics. As has been pointed out previously, this suppression of the haplophase is accentuated to an even greater degree in *Saccharomycodes ludwigii*, in which haploids rarely emerge as free cells from the ascus. On this basis, it is not surprising that methods which were successful in the case of *Schizosaccharomyces pombe*, in which the mutational potentialities of the haploids could be expressed, failed in the case of both *S. octosporus* and *Saccharomycodes ludwigii*.

*Isolation of unadaptable haploid strains from adaptable parent types.* Underlying the experiments on the relation of sporulation to adaptation was the implicit assumption that all that was required to break down the genetic stability of a diploid strain was the introduction of the haplophase. Although the validity of this assumption would in general not be questioned, it may be doubted insofar as variation in a particular direction is concerned. Thus it is conceivable that the haplophase of a particular strain might not contain within its mutational potentialities the ability to mutate in the direction of, for example, galactose fermentation. To answer this question as well as certain

others on possible origins of unadaptable types, experiments were undertaken to see if unadaptable haploid populations could be isolated.

The two *Saccharomyces cerevisiae* strains used were the objects of previous study. Strain Lk2G12 is a stable diploid which adapts itself to galactose fermentation within 80 minutes by direct interaction. Strain Db23B is a haploid which adapts itself through mutation. The other diploid strain employed, *S. carlsbergensis*, adapts itself directly within 90 minutes.

In the case of both diploid strains, the haplophases produced from them can exist independently and reproduce readily in the basic 8 per cent glucose medium. The experimental method used for the two diploid strains was the same. Heavy sporulation was induced by the methods already described. The mixture of spores and cells was heavily inoculated into 300 ml of basic 8 per cent glucose broth medium. Samples were removed at intervals early in the growth period and streaked on plates. The usual morphological and physiological variants which accompany haplophase-containing cultures made their appearance. The different kinds of clones were then inoculated into the

TABLE 2

*Physiological characteristics of the three unadaptable strains*

$Q_{O_2}$ ,  $Q_{CO_2}$ ,  $Q_{CO_2}^N$  are all calculated on the basis of gas consumed or evolved per mg dry weight per hour. Each figure is the average of three determinations on 48-hour galactose-grown cultures.

Strain	WITHOUT GALACTOSE				GALACTOSE ADDED			
	$Q_{O_2}$	$Q_{CO_2}^O$	$Q_{CO_2}^N$	R.Q.	$Q_{O_2}$	$Q_{CO_2}^O$	$Q_{CO_2}^N$	R.Q.
LN	8.5	8.6	0.02	1.01	15.4	15.4	0.03	1.00
CN	11.6	11.5	0.01	0.99	23.0	22.7	0.10	0.99
DN	5.4	5.4	0.00	1.00	12.5	12.7	0.12	1.02

basic broth medium containing 8 per cent galactose. These were incubated and their galactose fermentation characteristics followed. Some were obvious fermenters, as evidenced by copious gas evolution on shaking the culture flasks, and were discarded. Others, however, had to be examined manometrically to be certain of the presence or absence of fermentation. Using this procedure the authors examined 31 clones stemming from LK2G12 before a nonfermenting strain (LN) was isolated. Two out of the 31 entirely failed to grow on galactose and were discarded since they could not be used to test the permanency of the galactose nonutilization character. The strain LN grew perfectly well on galactose, apparently utilizing it by a purely aerobic mechanism. The R.Q. in three measurements averaged 1.01 and no galactose disappeared under nitrogen. The physiological characteristics of LN and the other unadaptable haploid strains are summarized in table 2. The LN strain was carried on 8 per cent galactose medium, transferred every 4 days for 5 months. It was tested weekly, and later twice weekly, for evidence of anaerobic  $CO_2$  production from galactose, always with negative results. That it is a haploid strain is

demonstrated by the predominant small, round cells observed in wet mounts and, more important, by its instability. The latter character is shown by figure 2, which represents a streak plate made from one of the routine galactose broth cultures. Smooth, micro-type colonies may be observed. Occasionally, rough variants may be noted. The very small colonies are physiological as well as morphological variants. Their growth rates are much lower than those of the other haploids, and the individual cells are approximately  $\frac{1}{4}$  of the normal size.

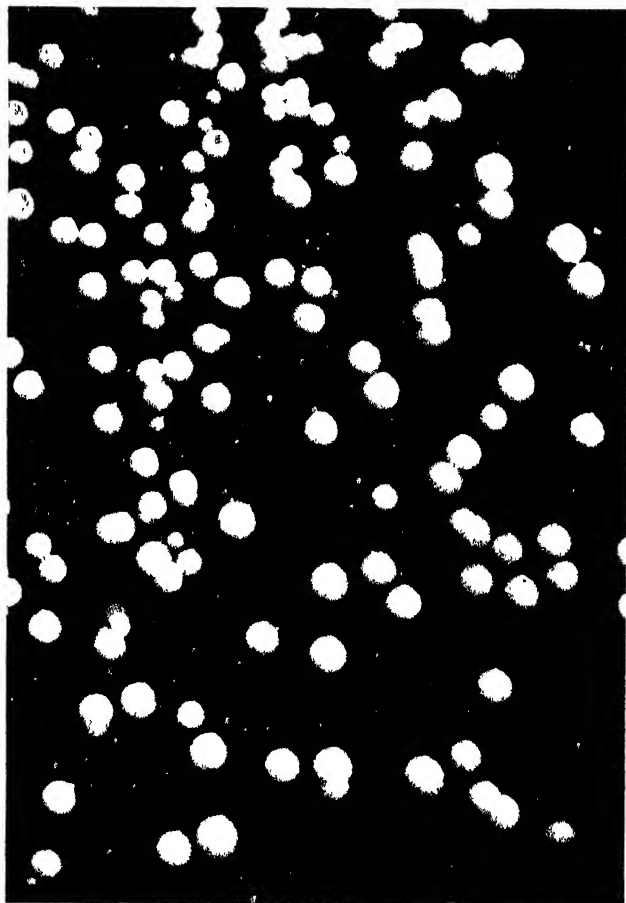


FIG. 2. STREAK PLATE DEMONSTRATING GENETIC INSTABILITY OF LN, AN UNADAPTABLE HAPLOID STRAIN OF *SACCHAROMYCES CEREVISIAE*

In the case of *Saccharomyces carlsbergensis*, the examination of 22 clones led to the isolation of a nonfermenting haploid strain (CN) which, on subsequent examination by the same methods employed with LN, did not revert to the fermenting type. Table 2 gives its metabolic characteristics on galactose. None of the 22 clones failed to grow on galactose. Four of the 22 clones when first isolated were nonfermenters when tested manometrically, but reverted

within 2 weeks to the fermenting type when subcultured on galactose. Strain C'N, however, has retained its nonfermenting character for 4 months, despite its obvious colonial instability as evidenced by figure 3, which represents a streak plate from a routine galactose broth culture. Here a relatively small rough type, a smooth type, and the micro-types are represented.

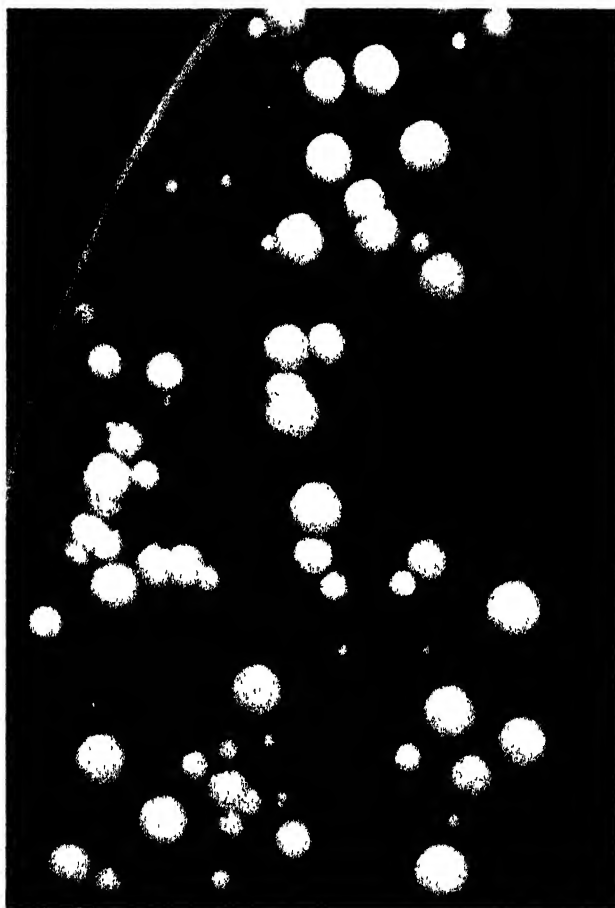


FIG. 3. STREAK PLATE DEMONSTRATING GENETIC INSTABILITY OF C'N, AN UNADAPTABLE HAPLOID STRAIN OF *SACCHAROMYCES CARLSBERGENSIS*

In the experiments with Db23B the problem of getting variant types was simpler since it was a haploid strain. It was seeded into the glucose medium, and streak plates were made from which clones were selected for testing. The fifth clone tested (DN) behaved in the same way as both LN and C'N. One of the 5 did not grow on galactose, whereas the other 3 could mutate to the adaptable type. Strain DN was carried in the usual manner in 8 per cent galactose for 4 months without showing any ability to produce  $\text{CO}_2$  anaerobically from galactose (table 2). It is also an unstable strain as is shown by figure 4

which represents a streak plate from a galactose culture. Here the rough and smooth forms predominate, although a small rough form may also be seen.

#### DISCUSSION

The relative significance of the experiments reported may be best evaluated in terms of the life cycle of yeasts as it is understood to date. Figure 5 is a diagrammatic representation of the life cycle and also indicates sources of variation at various stages. Starting with diploid cells, we find that these may sporulate and give rise to haploid segregants. These segregants may be and often are strikingly different from the parent types; the extent of the difference would depend on the degree of heterozygosis of the parent diploid.

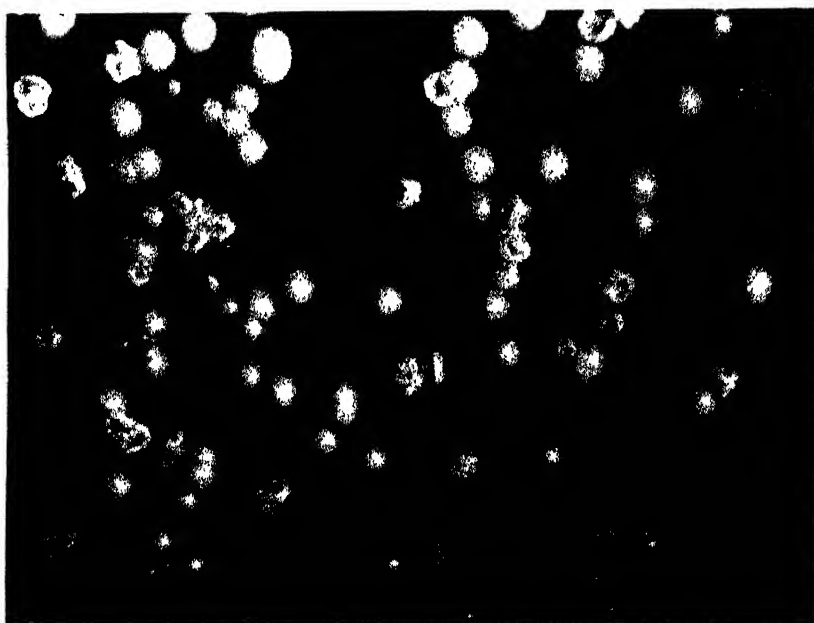


FIG. 4. STREAK PLATE DEMONSTRATING GENETIC INSTABILITY OF DN, AN UNADAPTABLE HAPLOID STRAIN OF *SACCHAROMYCES CEREVISIAE*

Thus, in a recent investigation (Lindegren, Spiegelman, and Lindgren, 1944) into the mechanism of the inheritance of adaptation to melibiose fermentation, it was possible to demonstrate simple Mendelian segregation of the adaptable character from a heterozygotic diploid parent.

The haploid segregants originating from the ascus may, if of suitable mating types, copulate to produce new diploid cells. These may or may not differ from the parent type depending on the nature of the recombination. On the other hand, if the haploids do not copulate they can in some strains exist as such by continued vegetative divisions, during which they usually give rise to mutants of various types. In general, it has been found that the mutational variants have lost the ability to copulate, probably owing to concomitant disturbances modify-







ing the effects of the gene which differentiates the mating type. This results in the maintenance of the haplophase nature of the culture.

From the point of view of the life cycle it seems clear why the experiments reported here were successful in adapting *Schizosaccharomyces pombe*, whereas previous ones had failed. Two conditions were satisfied in the present case that were not satisfied simultaneously in earlier attempts. One is a massive inoculation of heavily sporulating cultures which provides a source for a large

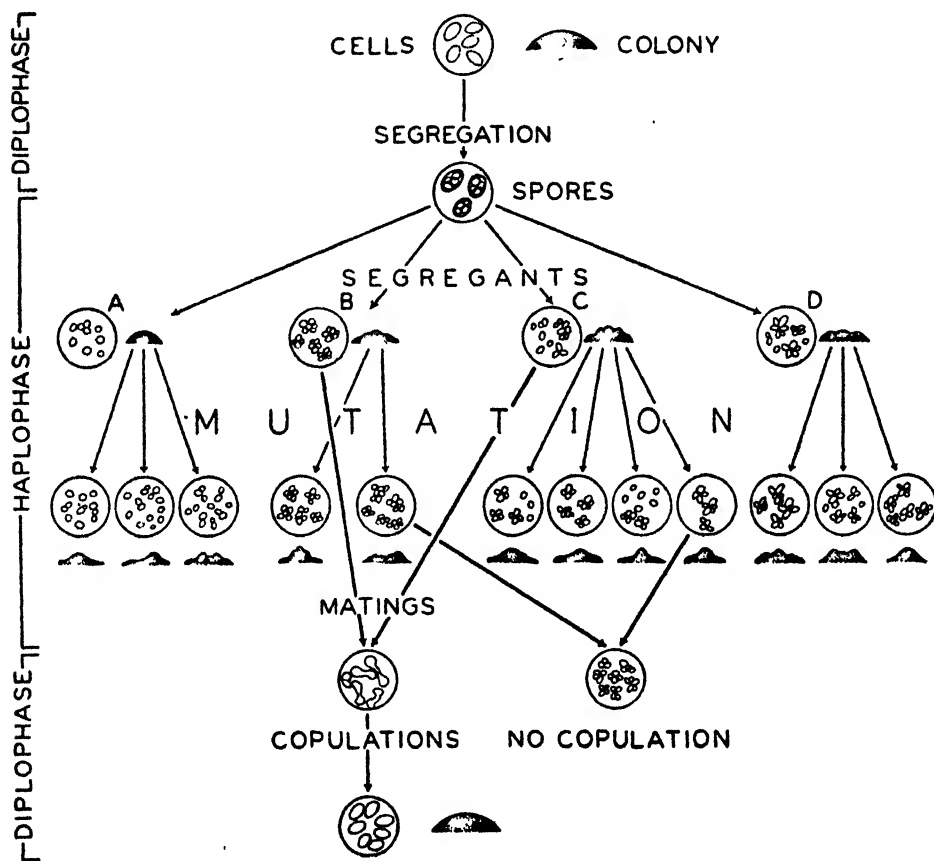


FIG. 5. LIFE CYCLE OF A SPOROGENOUS YEAST SHOWING SOURCES OF GENETIC VARIATION

number of various haploid segregants. The second was the inclusion of enough glucose in the adapting medium to allow the asci to germinate and the haploids to go through a relatively large number of divisions before intensive selection for the galactose fermenters took place. Under these conditions the heterogeneity of the haplophase originating from the segregation is further increased by the ability of the members to express their mutational potentialities. The chances for obtaining mutants of the desired nature is thus greatly augmented.

The experiments on the isolation of the three unadaptable variant haploid

strains from adaptable parents suggests a possible explanation for Wise and Appling's (1944) finding on the loss of galactose fermentability by a strain of *Saccharomyces cerevisiae*. Some cultures sporulate readily on agar slants, and it is probable that the variable haplophase was introduced from the stock strain. One interesting aspect of the three unadaptable strains is their inability, despite their demonstrated genetic instability, to mutate in the direction of galactose fermentation. While no absolute statement can be made, it must be recalled that the generation time of these organisms is but a little over an hour. Consequently, these experiments have extended over 1,200 generations involving billions of individuals in each generation. All this has been going on under conditions where intense selection of the fermenting type is favored. Thus if the back mutation should occur, it either does so with a vanishingly small frequency, or it may be accompanied by secondary lethal effects.

These experiments then would indicate that whereas sporulation is a necessary condition for populational adaptation by mutation of a diploid strain, it may not be sufficient for two reasons: (1) a haplophase suppression mechanism may exist as in *Schizosaccharomyces octosporus* and *Saccharomyces ludwigii* or (2) the haploids may not possess the capacity to mutate in the direction sought.

The wide variety of physiological variants which may be isolated from a single strain once the haplophase is introduced makes it difficult to characterize yeast types by their biochemical properties. In the present study closely related forms stemming from the same parent may or may not possess the ability to ferment galactose. Under these circumstances it is difficult to justify placing much weight on fermentation characteristics in classification schemes. This extreme heterogeneity is present in even the highly inbred strains as the baking yeasts which have been intensely selected for high aerobic CO<sub>2</sub> production on sucrose over a number of years. A recent study (to be discussed elsewhere) of 12 baking strains has demonstrated that they all can give rise to progenies having physiological properties diametrically opposed to those for which the parent type was selected.

In any case it is hopeless to attempt to characterize a yeast strain by its biochemical or morphological properties unless it is a diploid and care is taken to prevent its sporulation.

#### SUMMARY

*Schizosaccharomyces pombe*, previously reported as unadaptable, has been adapted to galactose fermentation by inoculation with heavily sporulating cultures into 2 per cent glucose 8 per cent galactose mixtures. The same methods failed with both *Schizosaccharomyces octosporus* and *Saccharomyces ludwigii*.

These results are interpreted in terms of the genetic instability of the haplophase and its suppression by rapid copulation in the latter two strains.

Unadaptable haploid strains have been isolated from adaptable parents. The significance of this for an understanding of the range of biochemical variation of mutant types is discussed.

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# SOME EFFECTS OF INDUCED STREPTOTHRICIN RESISTANCE ON LACTOBACILLUS CASEI<sup>1</sup>

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Streptothricin, the antibiotic produced by *Actinomyces lavendulae* (Waksman, 1943; Woodruff and Foster, 1943), has been reported to inhibit many organisms including *Lactobacillus casei* (Waksman and Woodruff, 1942). McKee and Houck (1943) and others have shown that when susceptible organisms are grown in the presence of slightly smaller concentrations of inhibitory antibiotics than those that prevent growth, resistance to these antibiotics is acquired. Since the growth factor requirements for *L. casei*, as well as its general bacteriology and biochemistry, are comparatively well defined, an investigation was undertaken to determine whether any marked differences existed between the resistant cultures and the susceptible parent culture.

## EXPERIMENTAL

Resistance to streptothricin was induced by growing the culture of *L. casei* (A.T.C.C. 7469) for several successive transfers at short intervals in tubes of glucose yeast extract broth containing increasing amounts of a crude preparation of streptothricin.<sup>2</sup> The first tube which contained 5 *Escherichia coli* units per ml was inoculated with a loop of 1 to 100 dilution of a culture 48 hours old which had been grown on a glucose yeast extract tryptone broth. After 24 hours of incubation at 37 C this tube was used to inoculate a second tube containing 11 *E. coli* units per ml. Good growth was obtained in this tube after 12 hours of incubation. When a loop transfer was made to a third tube containing 41 *E. coli* units per ml, however, good growth was not observed until after 36 hours of incubation. A second transfer was made at this streptothricin level, and good growth was obtained after 12 hours of incubation. A loop of this last tube was plated on glucose yeast extract agar, and ten isolated colonies were picked for further study. These cultures were still resistant at the 41 *E. coli* units per ml level 3 months after the original isolation.

The growth of the parent and the resistant strains was compared on nutrient broth containing various carbon sources as directed in the *Manual of Methods for the Pure Culture Study of Bacteria*. No distinct differences were observed between the different strains in these tests. These observations were checked by growing the strains on the amino acid vitamin medium developed by Hutchings (Hutchings and Peterson, 1943). The carbon source in question was

<sup>1</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

<sup>2</sup> We are indebted to Dr. S. A. Waksman for a supply of a streptothricin preparation, containing 500 *E. coli* units per ml.

substituted for glucose at a 2 per cent level in the medium. The growth of the strains was also compared on various carbon sources by growing them on the riboflavin assay basal medium with added excess riboflavin as described by Strong and Carpenter (1942). In this medium, also, the carbon sources in question were substituted for the glucose at a 2 per cent level. The growth on these media was observed by measuring the turbidity and the acid production during a 72-hour incubation period. The acid production results are presented in table 1.

The lactic acid produced by these cultures on a medium consisting of 5 per cent glucose, 3 per cent malt sprouts, and 1 per cent calcium carbonate was isolated after a fermentation period of two weeks (incubation at 37 C). The zinc salt was prepared and its properties compared with that described by Tatum and Peterson (1935). All the cultures produced *d*-lactic acid, as indicated by the optical rotation and the water of hydration of the zinc salt.

TABLE 1  
*Growth on various carbon sources*

CULTURE	ML 0.1 N ACID FORMED PER 10 ML OF MEDIUM														
	Glucose		Raffinose		Mannitol		Sucrose		Xylose		Glycerol		None		Lactose
	A*	B†	A	B	A	B	A	B	A	B	A	B	A	B	B
Parent.....	9.8	11.2	1.0	1.6	3.3	7.7	0.9	1.6	1.9	2.4	1.1	1.9	0.6	0.9	10.9
Isolate 1.....	10.2	11.2	1.8	1.7	3.3	8.8	1.5	2.5	1.3	1.7	0.8	0.9	0.8	0.9	11.6
Isolate 3.....	10.6	12.0	0.9	1.8	3.9	7.8	0.8	1.6	2.0	2.4	1.3	1.6	0.8	0.9	11.5
Isolate 10.....	10.0	11.9	0.7	1.6	2.8	8.7	0.8	2.0	1.6	2.2	0.9	1.6	0.4	0.9	12.1
Uninoculated medium.....	0.2	0.7	0.2	0.8	0.2	0.7	0.2	0.7	0.6	1.1	0.3	0.7	0.2	0.7	0.7

\* Synthetic medium (Hutchings and Peterson, 1943).

† Riboflavin basal medium with 4 µg of added riboflavin per 10 ml (Strong and Carpenter, 1942).

All the cultures grew equally well in glucose nutrient broth at an initial pH of 7.5 (growth measured turbidimetrically). No great differences in growth were observed between the cultures when they were grown in glucose yeast extract broth containing 2.5 per cent or 5 per cent sodium chloride.

Growth at various temperatures of incubation on a glucose yeast extract sodium acetate medium was studied. The acid production after 72 hours of incubation was determined. The titration data are presented in table 2.

The strains were grown on the riboflavin, pantothenic acid, and biotin assay media (Strong and Carpenter, 1942; Neal and Strong, 1943; Shull, Hutchings, and Peterson, 1942) at various levels of the respective vitamins. Indicative results are presented in table 3. The titration figures in this and the preceding tables are the averages from at least two tubes, and the majority of the experiments were repeated three times.

The results of these experiments seemed to indicate that some differences

existed between the strains. The stability of these differences was tested by two methods. First, the strains were transferred on the riboflavin assay basal

TABLE 2  
*Growth at various temperatures*

CULTURE	ML 0.1 N ACID FORMED PER 10 ML MEDIUM INCUBATED AT:														
	12.5 C*			15 C*			24 C			30 C			40 C		
	A†	B‡	C§	A	B	C	A	B	C	A	B	C	A	B	C
Parent.....	2.4	5.8	5.6	4.6	5.0	4.3	9.8	10.1	10.0	9.6	11.5	11.2	9.8	11.3	10.3
isolate 1.....	2.1	5.3	6.3	5.4	3.1	3.5	9.8	10.8	11.4	9.5	11.1	11.8	1.9	7.6	6.9
isolate 2.....	2.1	6.4	5.7	6.1	4.0	4.5	9.3	9.5	9.2	9.8	10.8	11.4	3.2	9.8	4.5
isolate 3.....	1.0	5.1	5.6	5.5	5.0	4.7	9.8	9.5	9.7	9.4	11.1	11.2	2.5	5.0	4.2
isolate 4.....	1.4	6.5	7.0	5.1	4.5	4.2	9.6	9.8	9.3	9.8	11.3	11.5	1.4	10.0	9.5
isolate 5.....	1.5	7.8	8.9	5.6	4.8	4.6	9.8	9.8	9.0	9.8	11.1	11.6	3.7	3.5	0.8
isolate 6.....	1.2	6.0	6.7	4.3	5.5	3.1	9.8	10.6	10.2	9.8	11.5	12.1	5.0	9.0	9.5
isolate 7.....	0.7	7.5	7.4	3.8	6.5	5.2	9.8	10.1	10.2	9.7	11.5	12.2	5.0	9.0	2.1
isolate 8.....	1.9	7.7	7.3	5.7	5.6	4.3	9.8	11.3	9.2	9.7	11.5	11.2	3.3	8.8	10.2
isolate 9.....	2.9	8.5	7.2	5.4	5.8	6.0	10.0	10.1	9.4	9.7	11.1	11.5	9.7	9.7	9.6
isolate 10.....	0.7	6.6	6.4	5.8	5.5	5.3	9.8	11.5	11.1	9.5	10.9	11.5	8.2	10.0	9.6

\* After 10 days of incubation; all others incubated for 72 hours.

† At time of isolation.

‡ Three months after isolation.

§ After 10 successive transfers at 24-hour intervals.

TABLE 3  
*Growth on various microbiological assay media*

CULTURE	ML 0.1 N ACID FORMED PER 10 ML OF MEDIUM CONTAINING INDICATED AMOUNT OF VITAMIN				
	0.02 µg calcium pantothenate			0.1 µg riboflavin	0.072 mg biotin
	A*	B†	C‡	A	A
Parent.....	3.0	3.8	4.0	5.1	3.9
Isolate 1.....	4.0	5.5	4.9	5.4	4.1
2.....	4.8	5.8	6.1	6.2	4.5
3.....	4.9	6.0	5.8	5.9	4.6
4.....	3.7	4.9	4.8	5.1	5.1
5.....	5.0	5.8	5.6	5.0	5.3
6.....	3.1	4.7	4.9	4.9	4.7
7.....	4.5	5.0	5.1	5.3	4.2
8.....	4.5	4.7	5.2	5.5	4.9
9.....	4.1	5.0	4.1	5.6	5.6
10.....	4.1	5.2	5.4	5.1	4.3

\* At time of isolation.

† Three months after isolation.

‡ After 10 successive transfers at 24-hr intervals.

medium, with added excess riboflavin, for ten successive times at 24-hour intervals. One-loop inocula were used for 10 ml of medium. At the end of this



period of transfers the washed cells of the tenth transfers were used as inocula in the pantothenic acid assay and in the temperature experiment. Second, the strains were stored for three months (without transfer) and were then used as sources of inocula for the pantothenic acid assay and for the temperature study. The results of these experiments are presented in the appropriate tables.

#### DISCUSSION

From the data presented in table 3 it appears that the streptothricin-resistant cultures of *L. casei* differ from the parent culture as to sensitivity to pantothenic acid and biotin, but not to riboflavin. From table 2 some differences are apparent in growth at various temperatures, as measured by acid production. Fermentation of the various carbon sources shown in table 1 may be considered to be about the same for each carbon source, although, of course, marked differences exist between the carbon sources.

The differences noticed in the B vitamin requirements and in the growth at various temperatures may not always appear to be consistent in certain experiments, but enough variation seems to exist between the strains to warrant the conclusion that rather permanent changes have taken place in the metabolism of the organism. The changes are called permanent, since they still existed after storage of the cultures and after rapid transfers on liquid media.

These results are somewhat different from those observed by other investigators who have studied penicillin-resistant strains of various organisms. The penicillin-resistant strains were said to be unchanged so far as carbohydrate fermentations and other bacteriological tests were concerned, but they had lost their virulent properties.

#### SUMMARY

Streptothricin-resistant strains of *Lactobacillus casei* (A.T.C.C. 7469) have been compared with the streptothricin-susceptible parent strain. Differences in the pantothenic acid and biotin sensitivity, as well as in growth at various temperatures, have been observed between the susceptible parent strain and the resistant strains. These differences seem to be permanent under the conditions of this study.

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# THE GERMICIDAL PROPERTIES OF CERTAIN QUARTERNARY AMMONIUM SALTS WITH SPECIAL REFERENCE TO CETYL-TRIMETHYL-AMMONIUM BROMIDE

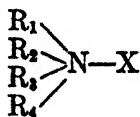
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In 1916 Jacobs, Heidelberger, and coworkers at the Rockefeller Institute reported the discovery of a new class of synthetic compounds which possessed potent bactericidal properties. By reacting hexamethylenetetramine with a large variety of substituted benzyl- or alkyl-halides, numerous quaternary salts of this base were obtained. It was observed that most of these salts had pronounced bactericidal properties. Unfortunately, these early observations received little attention.

It was not until 1935 that Domagk focused attention again on this group of compounds. This investigator observed that bactericidal properties are not restricted to the quaternary salts of hexamethylenetetramine. They are a general property of a large number of quaternary ammonium salts having the general formula:



According to Domagk, bactericidal properties are found in all compounds in which at least one radical (R) is a long chain aliphatic group ( $C_8H_{17}$  to  $C_{18}H_{37}$ ) either plain or substituted. The other three radicals (R) may be either one, two, or three lower alkyl- or benzyl-bound, or one aliphatically bound, phenyl radical. Two or three of them can even be arranged to form a cyclic compound as is the case in quaternary salts of hexamethylenetetramine, pyridine, piperidine, or morpholine. The radical -X is either a halide or another group, e.g., sulphate or acetate.

Shelton *et al.* (1939, 1940) studied a large number of quaternary ammonium salts derived from aliphatic or heterocyclic amines and observed that optimum bactericidal activity is present in compounds possessing one cetyl- ( $C_{18}H_{37}$ —) radical. The remaining three radicals (R) could be very simple; e.g., three methyl groups, as is the case in cetyl-trimethyl-ammonium bromide, or they could be arranged into a cyclic configuration, as is the case in cetyl-pyridinium chloride.

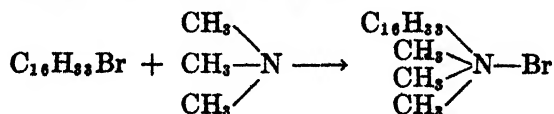
A wide variety of compounds, all within the broad field outlined by Domagk, have been synthesized since his studies were made; some of them have been studied in great detail. They are used extensively at present as antiseptics for surgical and gynecological procedures, such as skin and wound disinfection, and for sterilization of surgical instruments.

The germicidal properties of cetyl-pyridinium chloride have been studied by Blubaugh *et al.* (1939, 1941), Warren *et al.* (1942), and by Huyck (1944). Dunn (1936, 1937, 1938) studied the properties of a mixture of high molecular alkyl-dimethyl-benzyl-ammonium chlorides. From these and other studies it becomes evident that certain quaternary ammonium salts may be rated as the most potent bactericidal agents known at present. Their bactericidal action *in vitro* is far greater than that of the common antiseptics such as iodine, phenol, and mercurial compounds, and than that of most of the recently isolated antibiotics. They are active in high dilution against a large variety of microorganisms, gram-positive as well as gram-negative species, and fungi. They have proved their usefulness as antiseptics for preoperating skin sterilization.

The reports by Shelton *et al.* (1939, 1940) which show that maximum germicidal activity appears with the  $C_{16}H_{33}$ -substituted members of a homologous series of quaternary ammonium compounds suggest a more detailed study of the properties of cetyl-trimethyl-ammonium bromide. This choice has been further influenced by the practical consideration that solutions of this compound are being used at present extensively for skin disinfection and instrument sterilization. Barnes (1942) has shown that solutions of this compound, when applied to the skin, greatly reduce the normal skin flora and completely sterilize surgical equipment. This investigator further states that clinical experience has shown it to be painless and harmless when applied to the skin and that it is a very effective preoperative skin disinfectant. The suitability of cetyl-trimethyl-ammonium bromide for surgical purposes has also been studied by Williams *et al.* (1943) who found that a 1 per cent solution provides an excellent antiseptic medium for gloveless surgery as well as for treatment of infected wounds and burns. It has also been used for the treatment of impetigo by Forman (1943) and by Fischer (1944).

#### RESULTS

Cetyl-trimethyl-ammonium bromide may be prepared by the interaction of cetyl bromide ( $C_{16}H_{33}Br$ ) and trimethylamine.



It is purified by crystallization from organic solvents and is a white crystalline powder, decomposing and melting at 235 to 240 C. It possesses a somewhat astringent and slightly bitter taste.

It is soluble in alcohol, chloroform, and ethyleneglycol, but is very slightly soluble or insoluble in ether, petroleum ether, acetone, ethylacetate, benzene, and glycerol. Its peculiar solubility curve in water at different temperatures is shown in figure 1. (Data for this curve were determined by F. J. Bandelin of these laboratories.)

As may be seen from figure 1, cetyl-trimethyl-ammonium bromide is rather insoluble at temperatures below 20 C (less than  $\frac{1}{2}$  per cent). However, at

slightly higher temperature solubility increases tremendously until at 30 C a solution of more than 40 per cent may be obtained.

It is precipitated from solution by certain alkaloid reagents including picric acid, mercuric chloride, and gold chloride; but not by tannic acid or gelatin.

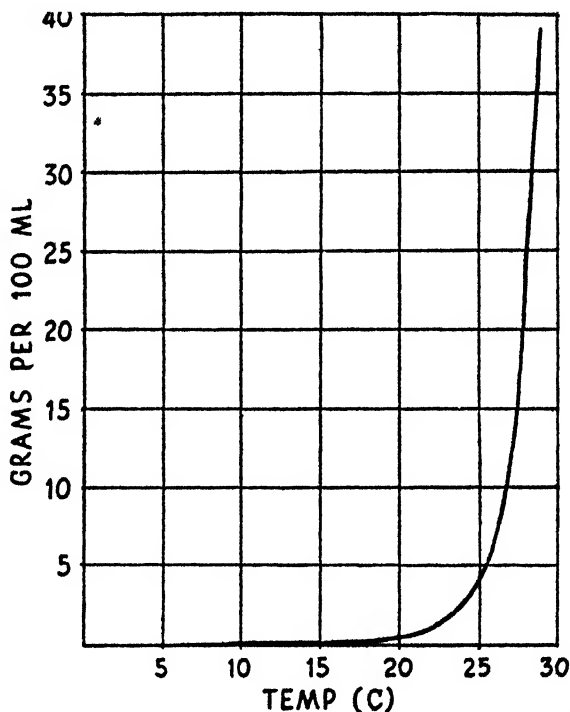


FIG. 1. SOLUBILITY OF CETYL-TRIMETHYL-AMMONIUM BROMIDE (IN GRAMS PER 100 ML) AT DIFFERENT TEMPERATURES FROM 5 C TO 30 C

TABLE 1

*Surface tension of solutions of cetyl-trimethyl-ammonium bromide*

CONCENTRATION	SURFACE TENSION
%	Dynes/cm
0	71.7
0.1	41.5
0.25	40.9
0.5	40.4
1.0	39.5
2.0	38.9

It is a cationic detergent and produces a considerable lowering of the surface tension. Values obtained at 26 to 27 C by the ring method with a deNouy tensiometer are given in table 1. (Data for this table were determined by L. L. Manchey, formerly of these laboratories.) Lowering of surface tension is recognized as a valuable property of antiseptic solutions, since the ability to

penetrate minute crevices and effect intimate contact between the germicide and the microorganisms depends to a large measure on this capacity.

The potency of a bactericidal substance can best be evaluated by determining the highest dilution that can bring about complete sterilization of a bacterial suspension under carefully maintained standardized conditions. For this purpose the technique proposed by the Food and Drug Administration and described in detail in U. S. D. A. Circular 198 (1931) is most frequently applied. By using this technique one can determine the maximum dilution of the bactericidal substance which produces sterilization of a 24-hour culture of the test organism in 10 minutes, but not in 5 minutes, under carefully controlled conditions, such as temperature, number of organisms, resistance of the strain used, and technique for determining complete sterilization. This dilution is defined as the "critical killing dilution" for the test organism used at a specified temperature. Similarly, the "critical killing time" of a germicidal solution is the minimum time required by such a solution to produce sterilization of a suspension of the test organism under the same carefully controlled conditions. However, valuable additional information may be obtained with a small amount of extra effort and strict adherence to the prescribed technique. By taking samples by loop at closer intervals and for a longer period than the 15 minutes suggested in the official FDA method, it is possible to determine the critical killing time for different concentrations of the disinfectant. The results obtained may be plotted on a curve and from this curve the critical killing time for any given concentration can be read. The critical killing dilution, in the sense of the FDA, can be read from this curve much more accurately than by direct determination.

The following technique was used: 2.5 ml of different dilutions of the disinfectant in distilled water were mixed with 2.5 ml of 1/15 molar phosphate buffer solution. To each tube was added 0.5 ml of a 24-hour culture of the test organism in Liebig bouillon (0.3 per cent Liebig beef extract and 0.5 per cent Armour special peptone). The resistance of the strain of *Staphylococcus aureus* and that of *Eberthella typhosa*, used for the experiment, was tested repeatedly against phenol and found to be within the limits set by the F.D.A.

In short intervals one loopful (about 0.01 ml) was transferred to fresh bouillon and after an incubation period of 48 hours the tubes were read for turbidity. The shortest time of exposure to produce sterilization was recorded for each concentration of disinfectant used. Each experiment was done at least twice and the average results were taken.

*Antiseptic properties of the homologues of cetyl-trimethyl-ammonium bromide (C<sub>16</sub>TAB)*

A study was made of the antiseptic properties of the homologous series of C<sub>n</sub>TAB, in which the cetyl radical (C<sub>16</sub>H<sub>33</sub>—) was replaced respectively by the groups: C<sub>8</sub>H<sub>17</sub>—, C<sub>10</sub>H<sub>21</sub>—, C<sub>12</sub>H<sub>25</sub>—, C<sub>14</sub>H<sub>29</sub>—, and C<sub>18</sub>H<sub>37</sub>—. (The author is indebted to Dr. R. H. Barry for preparing these quaternary ammonium salts.) These compounds will be referred to as C<sub>8</sub>TAB, C<sub>10</sub>TAB, etc.

The solubility of the members of the homologous series decreases rapidly with

the increase in carbon chain; that of  $C_{18}$ TAB in water at room temperature is less than 0.1 per cent and considerably less than that of  $C_{16}$ TAB.  $C_{14}$ TAB, on the other hand, is considerably more soluble than  $C_{16}$ TAB at room temperature. The lower derivatives are very hygroscopic.

All experiments were performed at 37 C and at a pH of 8.0. *S. aureus*, strain 209, was used as the test organism. It was found that complete sterilization was not achieved under the conditions of the experiment in one hour with 0.1 per cent solutions of compounds that had a chain length of 9 carbon atoms or less. However, as may be seen from figure 2, with increase in the

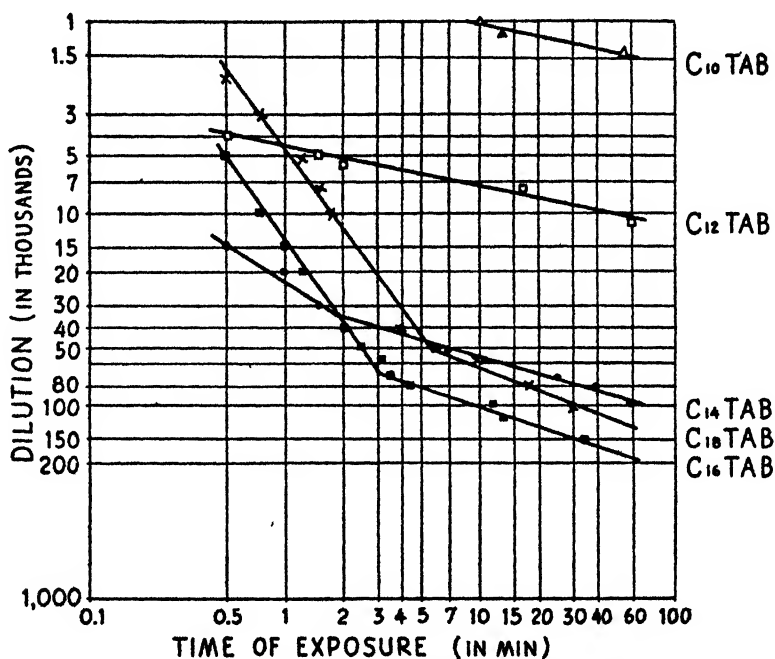


FIG. 2. CRITICAL KILLING TIME IN MINUTES FOR SUSPENSIONS OF STAPHYLOCOCCUS AUREUS AT 37 C AND pH 8.0, WITH DIFFERENT DILUTIONS OF THE GERMICIDES  $C_{10}$ TAB,  $C_{12}$ TAB,  $C_{14}$ TAB,  $C_{16}$ TAB, AND  $C_{18}$ TAB  
Logarithmic scale

chain length a tremendous increase in the germicidal potency of the compounds was observed. In accordance with the observation of Shelton *et al.* (1939, 1940), optimum activity is found in cetyl-trimethyl-ammonium bromide ( $C_{18}$ TAB). However,  $C_{14}$ TAB and  $C_{16}$ TAB are also very potent germicidal compounds. Rapid sterilization (within 5 minutes) is observed with  $C_{18}$ TAB in concentrations as low as 1/80,000 and a concentration of 1/150,000 will lead to complete sterilization within one hour.

By plotting the data on a logarithmic scale (figure 2), one may see that up to a concentration of 1:70,000 a straight line is obtained for  $C_{18}$ TAB, indicating that the rate of disinfection follows the law of a unimolecular reaction and that, therefore, the relation between the concentration and the time required to kill a



bacterial population can be expressed as  $C^n t = K$ . In this formula,  $C$  is the dilution,  $t$  the disinfection time, and  $n$  the dilution coefficient.

As may be seen, however, from figure 2, in dilutions greater than 1/70,000 the slope of the line changes markedly and the above formula cannot be used. This may be due to the fact that in higher dilutions we are not dealing with the bactericidal effect of the undissociated molecules, but with that of the ions. If this is the case, then the ionized molecule has a greater dilution coefficient than the nonionized molecule.

$C_{18}$ TAB gives a curve which is similar to that obtained for  $C_{16}$ TAB. Its bactericidal action is considerably slower than that of  $C_{16}$ TAB and it is not active in so high a dilution. The curve obtained with  $C_{14}$ TAB, on the other hand, lacks the pronounced bend of  $C_{16}$ TAB and  $C_{18}$ TAB. The slope of this curve as well as those for  $C_{12}$ TAB and  $C_{10}$ TAB might suggest that these compounds are largely ionized in the concentration used.  $C_{14}$ TAB is a faster-working disinfectant than cetyl-trimethyl-ammonium bromide, but it is not active in so high a dilution.

TABLE 2

*Bacteriostatic potency of a homologous series of quaternary salts against Staphylococcus aureus in Liebig bouillon, pH = 6.8, temp 37 C*

BACTERICIDAL AGENT	CRITICAL DILUTION PREVENTING GROWTH
$C_8$ TAB	1:1,500
$C_{10}$ TAB	1:7,000
$C_{12}$ TAB	1:70,000
$C_{14}$ TAB	1:340,000
$C_{16}$ TAB	1:400,000
$C_{18}$ TAB	1:400,000

The data presented refer to complete sterilization of a bacterial suspension. Preliminary experiments showed that although considerably lower concentrations of  $C_{16}$ TAB than indicated in the curve (about 1/500,000) do not cause complete sterilization, they do reduce the percentage of surviving bacteria to a mere fraction of the original number. Undoubtedly  $C_{16}$ TAB is, therefore, one of the most potent germicidal agents. Its phenol coefficient at 37 C and pH 8.0 is of the order of 1200.  $C_{14}$ TAB and  $C_{18}$ TAB are of comparable potency, but slightly below that of  $C_{16}$ TAB, each with a phenol coefficient of 750.

Similar results for the evaluation of the homologues were obtained using the dilution technique. Increasing dilutions of the bactericidal agents in Liebig broth of pH 6.8, inoculated with *S. aureus*, were made, and all tubes were incubated for 48 hours at 37 C. The highest dilution which prevented *S. aureus* from growing was recorded. This dilution is an expression for the bacteriostatic potency of the agents. The results obtained are given in table 2.

As may be seen from table 2,  $C_{14}$ TAB,  $C_{16}$ TAB, and  $C_{18}$ TAB prevent *S. aureus* from growing in dilutions of 2.5 to 3  $\mu$ g per ml. This is of the same order as several potent antibiotics, such as fumigacin or aspergillilic acid.

A third method, frequently used for evaluation of germicides, is the agar cup method or the "penicillinder" modification of it. Liebig nutrient agar plates, inoculated with *S. aureus*, are provided with small glass cylinder cups. Into each cup a few drops of a 0.1 per cent solution of the antiseptic are placed. The plates are then incubated for 24 hours and the width of the zone of inhibition around the cups measured. The results obtained are presented in table 3.

As may be seen from table 3, practically no activity is observed with the compounds C<sub>7</sub>TAB and C<sub>8</sub>TAB. The largest zone of inhibition is obtained with C<sub>12</sub>TAB, notwithstanding the fact that this compound is considerably less germicidal than C<sub>14</sub>TAB, C<sub>16</sub>TAB, and C<sub>18</sub>TAB. Undoubtedly this is due to the fact that C<sub>12</sub>TAB is able to diffuse more freely through agar than its higher homologues.

TABLE 3

*Bacteriostatic evaluation of a homologous series of quaternary salts by the agar cup method in Liebig bouillon agar, pH = 6.8, temp 37 C, with Staphylococcus aureus as a test organism*

BACTERICIDAL AGENT	ZONE OF INHIBITION	
	Complete	Partial
	mm	mm
C <sub>7</sub> TAB	0	0
C <sub>8</sub> TAB	0	1
C <sub>9</sub> TAB	0.5	4
C <sub>10</sub> TAB	1.5	6
C <sub>12</sub> TAB	5.5	5
C <sub>14</sub> TAB	4	1
C <sub>16</sub> TAB	0.5	1
C <sub>18</sub> TAB	0	0.5

This lack of correlation between the plate test and dilution tests was observed recently by Tobie and Ayres (1944) also. The use of the plate test for evaluation of certain germicides undoubtedly has serious drawbacks and should not be depended on unless other tests also show high germicidal activity.

*Influence of pH on the activity of cetyl-trimethyl-ammonium bromide*

Figure 3 represents the results of a series of tests on the germicidal potency of cetyl-trimethyl-ammonium bromide at different pH's. *S. aureus* was used as the test organism, and the experiments were conducted at a temperature of 37 C. Phosphate buffer (1/15 M) was used in all tests to maintain a constant pH. As may be seen from figure 3, cetyl-trimethyl-ammonium bromide is considerably more active at a high pH. At pH 8.0 C<sub>16</sub>TAB may be diluted considerably more than at pH 5 and still exhibit germicidal potency. Moreover, the time required to obtain complete sterilization is shorter at higher pH for all concentrations used. Even at pH 5, however, C<sub>18</sub>TAB is still a highly potent germicide.

*Influence of temperature on the activity of cetyl-trimethyl-ammonium bromide*

Similar experiments were done at a temperature of 20 C. The results obtained were similar to those obtained at 37 C. As would be expected, the bactericidal activity at 20 C is reduced as compared with that at 37 C. Critical killing dilutions (at 20 C) after 10 minutes' exposure at pH 5.0, 6.7, and 8.0, with *S. aureus* as the test organism, are 1:18,000, 1:34,000, and 1:50,000, respectively. At a temperature of 37 C these values are, respectively, 1:52,000, 1:80,000, and 1:100,000. The phenol coefficient for  $C_{16}$ TAB at 20 C at pH

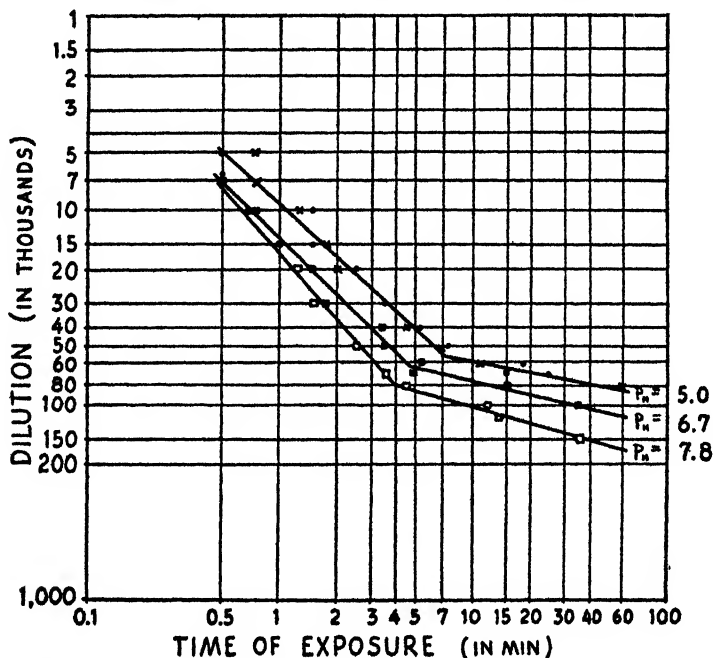


FIG. 3. CRITICAL KILLING TIME IN MINUTES OF  $C_{16}$ TAB FOR SUSPENSIONS OF *STAPHYLOCOCCUS AUREUS* AT 37 C AT DIFFERENT pH's  
Logarithmic scale

5.0, 6.7, and 8.0 were found to be 300, 500, and 830, respectively, as compared with 650, 1,000, and 1,250 at a temperature of 37 C.

*Susceptibility of various bacteria species*

Several species of microorganisms were cultivated for 5 successive transplants in Liebig broth and then subjected to a germicidal test at 37 C and pH 8.0. The results of these tests are given in figure 4.

As may be seen from figure 4, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella paratyphi* are about equally sensitive to the action of  $C_{16}$ TAB. *Eberthella typhosa* is slightly more susceptible, whereas a strain of *Staphylococcus albus* was the most susceptible; a concentration of  $C_{16}$ TAB of 1/300,000 sterilized a suspension of the latter organism in 30 minutes as compared with a 1/150,000 concentration for *S. aureus*. Spores of *Bacillus subtilis* were most

resistant to the action of  $C_{16}$ TAB. A spore suspension of this organism was prepared by suspending the growth of a 3-day-old agar slant in Liebig bouillon. Before the experiment, this suspension was heated for 10 minutes at 80 C to destroy vegetative cells.

A concentration of 1/5,000 was required for complete sterilization of a *B. subtilis* spore suspension after a 1-hour exposure, and a 1/2,000 solution to obtain the same results in  $\frac{1}{2}$  minute. Vegetative cells of *B. subtilis*, however, seem to be very sensitive to the action of  $C_{16}$ TAB in so far as its bacteriostatic effect is concerned. A dilution test with Liebig bouillon, inoculated with the same spore suspension as was used for the germicidal test, showed complete

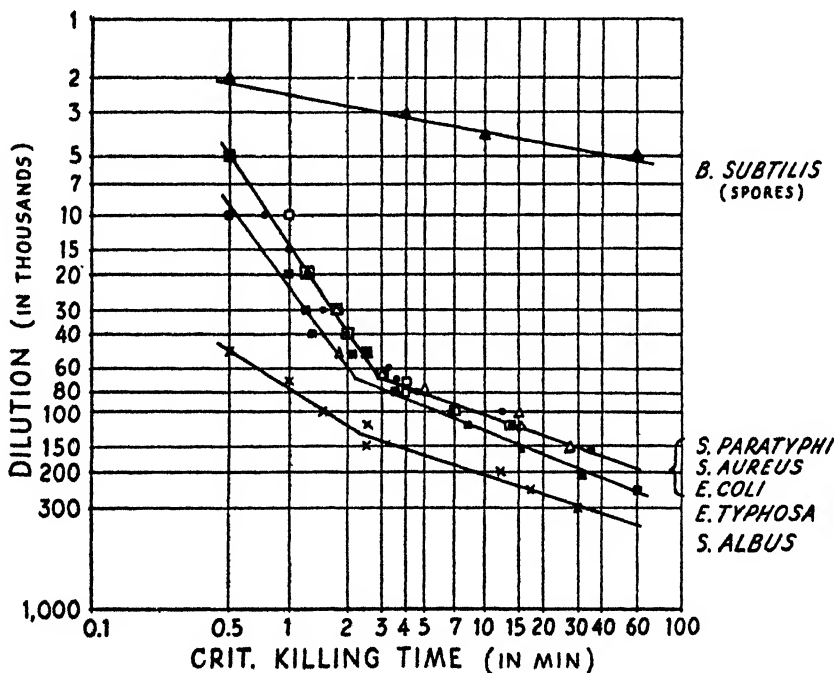


FIG. 4. CRITICAL KILLING TIME IN MINUTES OF  $C_{16}$ TAB AT 37 C AND pH 8.0 FOR SUSPENSIONS OF DIFFERENT BACTERIA

absence of growth in all tubes containing  $C_{16}$ TAB in a dilution of 1/700,000, or lower. This result is equal to or better than that obtained with *S. aureus*. It indicates that, although spores are not destroyed, small amounts of  $C_{16}$ TAB will prevent them from germinating or will destroy them when germination occurs.

#### *Influence of serum (and milk) on germicidal potency*

As is the case with most germicidal agents  $C_{16}$ TAB is inhibited in the presence of blood serum. Addition of  $C_{16}$ TAB to dilute serum causes a definite precipitation. With excess of serum, however, no immediate precipitate is formed. Figure 5 gives the results of a number of bactericidal tests with  $C_{16}$ TAB in the

presence of increasing amounts of normal horse serum. All tests were conducted at pH 8.0 and at 37 C with *S. aureus* as a test organism. As may be seen from figure 5, addition of as little as 2 per cent serum produces a marked reduction in the potency of C<sub>15</sub>TAB. In the presence of 2, 5, 10, and 20 per cent of serum, the phenol coefficient at 37 C decreases from 1,200 in the absence of serum to 380, 225, 150, and 62, respectively. Notwithstanding this serious inhibition, a 1:2,000 solution of C<sub>15</sub>TAB has still sufficient bactericidal potency left to sterilize a culture of *S. aureus* within  $\frac{1}{2}$  minute in the presence of 20 per cent serum.

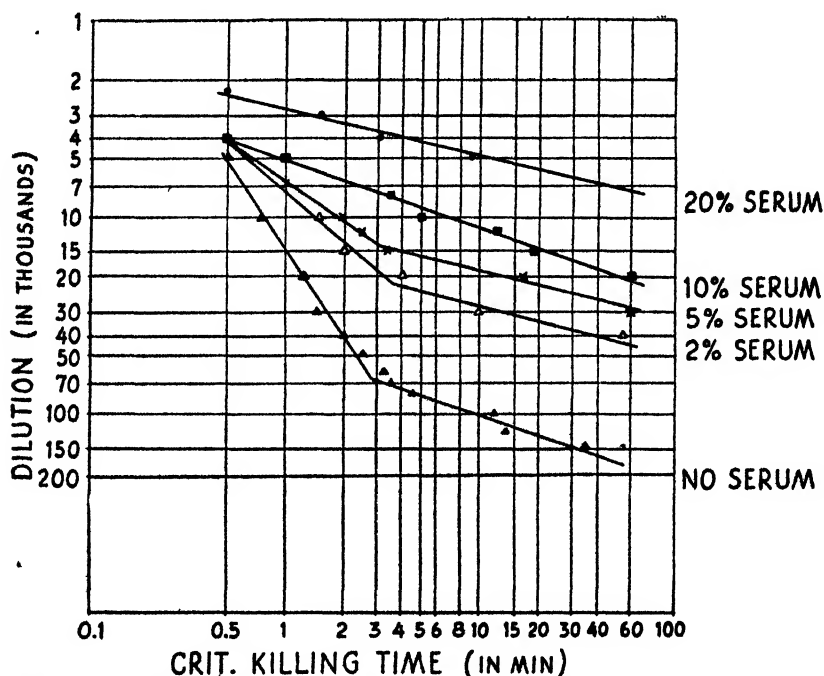


FIG. 5. CRITICAL KILLING TIME IN MINUTES OF C<sub>15</sub>TAB AT 37 C AND pH 8.0 FOR SUSPENSIONS OF STAPHYLOCOCCUS AUREUS IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF NORMAL HORSE SERUM

Milk also reduces the effectiveness of C<sub>15</sub>TAB considerably. In the presence of 50 per cent of milk the phenol coefficient for *S. aureus* at 37 C is reduced to 56.

The inhibitory effect of serum is less pronounced on the bacteriostatic potency of quaternary ammonium salts. Only 2 to 2.5 times as much of most of these compounds is required in the presence of 10 per cent serum to achieve the same inhibitory effect as under the same conditions without serum.

Table 4 gives the result of a comparative study of several of the common antiseptics, including certain quaternary ammonium salts, mercurials, and phenolic compounds, tested both in the presence and absence of serum.

Liebig broth, inoculated with *S. aureus*, and containing increasing amounts of the germicides, was incubated for 48 hours and the greatest dilution preventing growth was recorded.

As may be seen from table 4, Na-ethyl-mercuri-thiosalicylate ("merthiolate") is by far the most effective inhibitory agent, both in the presence and absence of serum. A dilution of one in two or three million will prevent *S. aureus* from growing. This action is mainly bacteriostatic, however, since even a hundred times higher concentration failed to sterilize the inoculated culture medium after an exposure of 24 hours to this germicide. By transferring a loopful to Brewer's medium, containing sodium thioglycollate, a substance which neutralizes the action of mercurials, the presence of surviving organisms could be readily demonstrated.

TABLE 4

*Bacteriostatic evaluation of germicides in Liebig bouillon, pH = 6.8, with and without serum, with Staphylococcus aureus as a test organism at 37 C*

BACTERICIDAL AGENT	CRITICAL BACTERIOSTATIC DILUTION	
	Liebig broth	Liebig broth + 10% serum
C <sub>10</sub> TAB.....	7,000	4,500
C <sub>12</sub> TAB.....	70,000	30,000
C <sub>14</sub> TAB.....	340,000	82,000
C <sub>16</sub> TAB.....	400,000	170,000
C <sub>18</sub> TAB.....	400,000	200,000
Mixture of high molecular alkyl-dimethyl-benzyl-ammonium chlorides .....	400,000	140,000
p-tertiary-octyl-phenoxy-ethoxy-ethyl-dimethyl-benzyl-ammonium chloride ..	400,000	140,000
Cetyl pyridinium chloride ..	1,000,000	420,000
Phenol.....	450	280
Hexylresorcinol..	30,000	6,800
Iodine.....	3,200	20,000
Na salt of 2:7 dibromo-4-hydroxy-mercuri-fluorescein.....	32,000	30,000
Na-ethyl-mercuri-thiosalicylate.....	2,300,000	3,000,000
4-nitro-anhydro-hydroxy-mercuri-o-cresol .....	750,000	470,000
Ag-protein (colloidal).....	18,000	16,000

All other compounds tested, including the quaternary ammonium salts, were mainly bactericidal. A concentration of 2 to 5 times the critical bacteriostatic dilution was found to be bactericidal. The inhibitory effect of serum on the activity of quaternary salts is approximately the same for all compounds tested; about 30 to 50 per cent of the activity remains effective in the presence of 10 per cent serum. This inhibition is of the same order or less than that observed with other germicides such as "metaphene" and hexylresorcinol.

A remarkable potency was observed with iodine in the presence of serum, far exceeding that obtained in the absence of serum. This additional activity, probably due to the interaction of iodine on serum constituents, was found to be of bacteriostatic nature only.

## SUMMARY

A study was made of the bactericidal and bacteriostatic properties of the homologous series of quaternary ammonium salts derived from tetramethyl ammonium bromide. It was found that bactericidal properties became evident when one methyl group was replaced by a nonyl group. Further increase in the chain length produced compounds of high germicidal potency with a definite maximum for cetyl-trimethyl-ammonium bromide. The effect of pH, temperature, and the inhibitory effect of serum on the bactericidal and bacteriostatic properties of this compound was studied in more detail. The bactericidal potency of this compound increases considerably with increasing pH. At pH 8.0 its phenol coefficient for *Staphylococcus aureus* at 37 C was found to be 1,200. When added to nutrient broth, 3  $\mu$ g per ml will prevent growth of *Staphylococcus aureus*. Gram-positive and gram-negative species are equally affected. In the presence of serum a considerable part of the germicidal potency is lost. Comparison of its potency with that of a series of commonly used disinfectants shows that this compound is one of the outstanding bactericidal and bacteriostatic agents.

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# CONVERSION OF DESTHIOBIOTIN INTO BIOTIN OR BIOTINLIKE SUBSTANCES BY SOME MICROORGANISMS<sup>1</sup>

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Dittmer, Melville, and Du Vigneaud (1944) used *Lactobacillus casei* as a test organism to demonstrate that *Saccharomyces cerevisiae*, strain 139, converted desthiobiotin into biotin or some biotin vitamer. Simultaneously Lilly and Leonian (1944a) reported that *Lactobacillus arabinosus*, *Sordaria fimicola*, and *Ceratostomella pini* 416 manifested increased growth when the nutrient medium contained less than depressant amounts of desthiobiotin in addition to biotin. Since none of these organisms grew in the presence of desthiobiotin alone, it was presumed that the increased growth was caused by the utilization of desthiobiotin in the presence of small amounts of biotin. It is possible that desthiobiotin might exert a sparing action on biotin under certain conditions.

Dittmer, Du Vigneaud, György, and Rose (1944) found that biotin sulfone supported only limited growth of *S. cerevisiae*, strain 139, and postulated that this vitamer of biotin can fulfill only a portion of the functions of biotin in the growth of this strain of yeast. These results suggested the possibility that organisms unable to use desthiobiotin in the absence of biotin may do so to a limited extent in its presence either because of partial replacement or because of transformation into other vitamers of biotin.

## QUALITATIVE STUDIES

Some preliminary tests were made with the following twelve yeasts: *Saccharomyces cerevisiae*, "old process," *Mycoderma valida*, *Zygosaccharomyces marxianus*, *Debaryomyces matruchoti* v. *subglobosus*, *Saccharomyces carlsbergensis*, *S. chodati*, *Endomycopsis fibuliger*, *Schizosaccharomyces pombe*, *Zygosaccharomyces lactis* 27, *Mycotorula lactis* 130, and *Torula lactosa* 168. These organisms were grown in a nutrient medium where desthiobiotin<sup>2</sup> (0.5 µg per liter) replaced biotin. After an incubation period of three days at 25 C the cells were harvested and hydrolyzed by autoclaving for one hour in 1 per cent sulfuric acid. The hydrolyzates were tested for biotin activity by *Lactobacillus arabinosus*, *Lactobacillus casei*, *Rhizobium trifolii* 205, and *Sordaria fimicola*. All four of these organisms grew well in all the hydrolyzates, indicating conversion of desthiobiotin into biotin or some biotin vitamer by all twelve of the foregoing yeasts.

Four filamentous fungi, *Neurospora crassa*, *Neurospora sitophila*, *Ceratostomella ips* 438, and *Ceratostomella montium*, were also grown in the presence of

<sup>1</sup> Published with the approval of the Director of the West Virginia Agricultural Experiment Station as Scientific Paper No. 334.

<sup>2</sup> Obtained through the courtesy of Dr. R. T. Major of Merck and Company.

desthiobiotin in place of biotin; the mycelium was acid-hydrolyzed, and the hydrolyzate tested with *Lactobacillus casei* and *Sordaria fimicola*. The ensuing rich growth indicated that these filamentous fungi also were capable of converting desthiobiotin into biotin, or some biotin vitamer active for these test organisms.

#### QUANTITATIVE STUDIES

The following four organisms were chosen for the quantitative studies: *Saccharomyces cerevisiae* "old process," *Neurospora sitophila*, *Sordaria fimicola*, and *Ceratostomella pini* 416. The first two grow just as well in the presence of desthiobiotin as in that of biotin; the last two make no growth when desthiobiotin replaces biotin in the medium. With the exception of the vitamins, the basal medium was essentially the same for all four organisms and consisted of the following substances:

KH <sub>2</sub> PO <sub>4</sub> .....	1 g
MgSO <sub>4</sub> .....	0.5 g
Casein hydrolyzate equivalent to.....	2 g of casein
Fumaric acid.....	1.32 g
Sodium carbonate.....	1.12 g
Glucose.....	25 g
Fe.....	0.0002 g
Zn.....	0.0002 g
Mn.....	0.0001 g
Distilled water.....	1,000 ml

The glucose, fumaric acid, and casein hydrolyzate were treated with norit A, 5 grams per liter.

The only vitamin added to the *Sordaria* medium was biotin, 4 micrograms per liter. The pH was adjusted to 6.5 before autoclaving. The incubation period was 6 days at 25 C. The *Neurospora* medium contained 100 micrograms of pyridoxine, 200 micrograms of thiamin, and 1 microgram of biotin. The pH was 5.5, and the incubation period 3 days at 25 C. The yeast medium contained 100 micrograms each of pyridoxine and pantothenic acid, 1 microgram of biotin, and 10 milligrams of inositol. The pH was adjusted to 5.0, and the incubation period was 3 days at 25 C. To each lot of 1,000 ml of *Ceratostomella* medium were added 200 micrograms each of thiamin, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, *p*-aminobenzoic acid, 5 micrograms of biotin, 10 mg of inositol, and the filtrate from 1 g of yeast extract twice treated with norit A at pH 4. In addition, 100 mg each of cystine and tryptophane were added to compensate for the possible shortage of these amino acids in the casein hydrolyzate. The pH of the medium was 6.0 before autoclaving. The incubation period was 8 days at 25 C.

The nutrient medium for each organism was divided into two lots: The first lot contained, in addition to the other vitamins, biotin in the amounts specified. The second lot contained biotin plus 1,000 micrograms of desthiobiotin per 1,000 ml of the medium. After the specified incubation period the yeast cells and the

fungus mycelium were separated from the spent medium by filtering through alundum crucibles. The filtrate was concentrated under reduced pressure to such an extent that upon the addition of 20 per cent of 95 per cent alcohol the volume was restored to the original level; this was stored in the refrigerator. The yeast cells and the fungus hyphae were acid-hydrolyzed, made into a definite volume with 20 per cent alcohol, and stored in the refrigerator.

#### ASSAY METHODS

Various amounts of both the filtrate and hydrolyzate were added to the various basic media and assayed for both biotin and desthiobiotin by means of the following test organisms: *Lactobacillus arabinosus*, *Lactobacillus casei*, *Rhizobium trifolii*, and *Saccharomyces cerevisiae*, "Gebrüder Mayer." The nutrient medium for the two species of *Lactobacillus* was that of Landy and Dicken (1942) except that glucose was increased to 20 g per liter, sodium acetate to 20 g, and cystine to 0.2 g. *Rhizobium trifolii* was grown in a medium of the following composition:

KH <sub>2</sub> PO <sub>4</sub> .....	1.0 g
MgSO <sub>4</sub> .....	0.2 g
NaCl .....	0.2 g
FeSO <sub>4</sub> .....	0.01 g
KNO <sub>3</sub> .....	1.0 g
Sucrose.....	10.0 g
Thiamin hydrochloride.....	100 µg
Distilled water....	1,000 ml

The pH of the solution was adjusted to 7, and after being filtered, it was readjusted to the same point. The cultures were incubated for 72 hours at 30 C. Norit treatment was omitted because it induces poor growth. *Saccharomyces cerevisiae*, "Gebrüder Mayer," was grown in the yeast medium described above. For a full description of the methods of assay used see Leonian and Lilly (1945).

Three of the test organisms used for assay, *Lactobacillus arabinosus*, *Lactobacillus casei*, and *Rhizobium trifolii* 205, do not respond to desthiobiotin either as a growth substance or as an antibiotin at the concentrations used in these assays. Therefore the assay values obtained with these organisms do not include desthiobiotin; that other vitamers of biotin may be active for these organisms must be considered. Lilly and Leonian (1944b) found no difference in the quantitative response of *Saccharomyces cerevisiae*, "Gebrüder Mayer," to biotin and desthiobiotin under the conditions used for assay. Equal-weight mixtures of biotin and desthiobiotin permitted the same amount of growth as with either separately. Thus, the assay values obtained by the use of *S. cerevisiae*, "Gebrüder Mayer," represent the sum of the amounts of biotin and desthiobiotin present. In addition, the possibility of other active vitamers of biotin for this organism must be considered.

Table 1 gives the assay results. All values are given in terms of micrograms of biotin, desthiobiotin, or biotin vitamers detected in one liter of spent medium, and in the amount of cells or hyphae harvested from one liter of the medium.

TABLE 1

*Conversion of desthiobiotin into biotin or vitamers of biotin*

Amounts of biotin and desthiobiotin recovered from cells of 4 organisms grown in the presence of known amounts of biotin, and biotin and desthiobiotin.

TEST ORGANISM	BIOTIN RECOVERED			BIOTIN AND DESTHIOBIOTIN RECOVERED		
	Cells	Medium	Total	Cells	Medium	Total
	<i>Sordaria fimicola</i> assayed					
	(4 µg of biotin added)			(4 µg biotin and 1,000 µg of desthiobiotin added)		
<i>L. arabinosus</i> . . . . .	0.83	0.10	0.93	0.76	0.33	1.09
<i>L. casei</i> . . . . .	2.16	0.21	2.37	1.12	0.14	1.26
<i>R. trifolii</i> 205 . . . . .	0.78	0.24	1.02	0.64	1.60	2.24
<i>S. cerevisiae</i> , "Gebrüder Mayer" . . . . .	0.69	0.20	0.89	29.64	600.00	629.64
	<i>Saccharomyces cerevisiae</i> , "old process," assayed					
	(1 µg of biotin added)			(1 µg biotin and 1,000 µg of desthiobiotin added)		
<i>L. arabinosus</i> . . . . .	0.18	0.03	0.21	1.84	0.16	2.00
<i>L. casei</i> . . . . .	0.53	0.12	0.65	5.76	0.0	5.76
<i>R. trifolii</i> 205 . . . . .	0.072	0.01	0.082	0.32	0.013	0.333
<i>S. cerevisiae</i> , "Gebrüder Mayer" . . . . .	0.12	0.02	0.14	6.45	640.00	646.45
	<i>Neurospora sitophila</i> assayed					
	(1 µg of biotin added)			(1 µg biotin and 1,000 µg of desthiobiotin added)		
<i>L. arabinosus</i> . . . . .	0.20	0.14	0.34	1.84	3.60	5.44
<i>L. casei</i> . . . . .	0.35	0.04	0.39	2.86	2.32	5.18
<i>R. trifolii</i> 205 . . . . .	0.06	0.04	0.10	4.50	9.90	14.40
<i>S. cerevisiae</i> , "Gebrüder Mayer" . . . . .	0.12	0.02	0.14	2.58	560.00	562.58
	<i>Cerastostomella pini</i> assayed					
	(2 µg of biotin added)			(2 µg biotin and 1,000 µg of desthiobiotin added)		
<i>L. arabinosus</i> . . . . .	0.65	0.11	0.76	0.62	0.42	1.04
<i>L. casei</i> . . . . .	1.52	0.14	1.66	1.16	0.34	1.50
<i>R. trifolii</i> 205 . . . . .	0.31	0.02	0.33	0.66	0.45	1.11
<i>S. cerevisiae</i> , "Gebrüder Mayer" . . . . .	0.42	0.05	0.47	2.23	600.00	602.23

## DISCUSSION

Table 1 shows that when a biotin-requiring organism is grown in the presence of biotin, a certain amount is no longer recoverable. The amount of biotin recovered depends upon the organism first grown in the medium, and upon the test organism used to assay the spent medium and cells. If we assume the

unrecovered biotin to be the amount "used," the situation with regard to *Ceratostomella pini* 416 is as follows: this organism was grown upon a medium containing two micrograms of biotin per liter; the amount of biotin "used" depends upon the test organism used for assay. Thus, *L. arabinosus* assayed 1.24  $\mu\text{g}$ ; *L. casei*, 0.34  $\mu\text{g}$ ; *R. trifolii* 205, 1.67  $\mu\text{g}$ , and *S. cerevisiae*, "Gebrüder Mayer," 1.53  $\mu\text{g}$ . There is a five fold variation in the amount of biotin "used" by *C. pini* under a given set of conditions, depending upon the choice of test organism used to assay "biotin."

It seems to us, therefore, that the explanation of these results should be sought along the following lines of reasoning: the changes wrought on the biotin molecule by *C. pini* 416 during growth are such that only a little of the biotin activity is lost for *L. casei*. These same changes in the biotin molecule, however, are such that *R. trifolii* 205 can utilize only one fifth as much of the remaining biotin activity as does *L. casei*. Similarly each biotin-requiring organism makes its own pattern of change on the biotin molecule, which is reflected in the changing proportion of activity reported by the test organisms for each case studied.

The situation with regard to desthiobiotin is somewhat more complicated. The amount of desthiobiotin recovered in the filtrate amounted to about 60 per cent of the amount added. Apparently all four organisms destroyed the activity of desthiobiotin for *S. cerevisiae*, "Gebrüder Mayer," to an equal extent. A sparing effect of desthiobiotin on biotin "used" by *S. fimicola* and *C. pini* was observed when *L. arabinosus* and *R. trifolii* 205 were used as test organisms. When *L. casei* was the test organism, the opposite was found. No evidence was found to support the hypothesis that organisms unable to utilize desthiobiotin in the presence of biotin (*S. fimicola* and *C. pini*) converted it into biotin or any of its vitamers active for *L. arabinosus*, *L. casei*, or *R. trifolii* 205. It is apparent, however, that the mycelium of *S. fimicola* stored desthiobiotin, inasmuch as *S. cerevisiae*, "Gebrüder Mayer," assayed 29.64 micrograms of "biotin." That this stored "biotin" was probably desthiobiotin was shown by the expected destruction of activity on acid autoclaving (see Dittmer, Melville, and Du Vigneaud, 1944). The situation with regard to the organisms that utilize desthiobiotin (*S. cerevisiae*, "old process," and *N. sitophila*) presents a number of points of interest. First, with regard to the "biotin" content of the cells of *S. cerevisiae*, "old process," grown in the presence of 1  $\mu\text{g}$  of biotin and 1000  $\mu\text{g}$  of desthiobiotin, both *L. arabinosus* and *L. casei* (neither of which respond to desthiobiotin) assay more biotin than was originally in the medium, and we conclude, therefore, that some desthiobiotin was converted into biotin or vitamers active for these organisms. However, *R. trifolii* 205 did not give an assay value greater than the amount of biotin originally present, so it appears doubtful that *S. cerevisiae*, "Gebrüder Mayer," converted much desthiobiotin into biotin per se. An alternative explanation would be the formation of an inhibitory substance that depressed the assay value of *R. trifolii* 205.

When the mycelium of *N. sitophila* grown in the presence of 1  $\mu\text{g}$  of biotin and 1000  $\mu\text{g}$  of desthiobiotin was assayed for "biotin," the three test organisms which were unable to utilize desthiobiotin assayed more biotin (1  $\mu\text{g}$ ) than was

originally added to the medium. Indeed, *R. trifolii* 205 assayed more "biotin" than either *L. arabinosus* or *L. casei*. It should be noted that *N. sitophila* excreted substances having "biotin" activity into the medium; this is in contrast with the situation found with *S. cerevisiae*, "old process," in which the enhanced activity was found in the cells alone.

The situation reported here is not inconsistent with what is known regarding vitamers. Although many vitamers of biotin have been shown to possess only a small fraction of the activity of biotin itself (Dittmer and Du Vigneaud, 1944), one (desthiobiotin) has the same activity as biotin for *S. cerevisiae*, strain 139 (Dittmer, Melville, and Du Vigneaud, 1944), and *S. cerevisiae*, "Gebrüder Mayer" (Lilly and Leonian, 1944b). Whereas in general vitamers may be thought to be less active than the vitamin, such is not always the case as was shown by Snell (1944) for pyridoxal and pyridoxamine, which have activities ranging from 1 to 6,000 depending upon the test organism in question.

To the important concept of species specificity of vitamers stressed by Burke and Winzler (1943) there must be added the equally important findings that vitamer activity may vary from test organism to test organism. With these two criteria in view the results presented here are susceptible of reasonable explanation. The complete exposition awaits further investigation.

#### SUMMARY

Twelve yeasts and four filamentous fungi grown upon desthiobiotin converted it into biotin or vitamers of biotin active for the following organisms unable to utilize desthiobiotin: *Lactobacillus arabinosus*, *Lactobacillus casei*, *Rhizobium trifolii* 205, and *Sordaria fimicola*. Quantitative determinations failed to recover all the biotin activity from the following organisms grown upon known amounts of biotin: *Sordaria fimicola*, *Saccharomyces cerevisiae*, "old process," *Neurospora sitophila*, and *Ceratostomella pini*. The amount of biotin activity recovered depended upon the organism being assayed and the test organism used.

When these organisms were grown in the presence of 1,000 micrograms of desthiobiotin in addition to biotin, *S. cerevisiae*, "old process," was found to convert desthiobiotin into substances having biotin activity for *L. arabinosus* and *L. casei*, but not for *R. trifolii* 205, whereas *N. sitophila* converted desthiobiotin into vitamers of biotin active for the former two test organisms and for *R. trifolii* 205 as well. No evidence of conversion of desthiobiotin into biotin vitamers active for *L. arabinosus*, *L. casei*, and *R. trifolii* 205 by *S. fimicola* and *C. pini* was found. The mycelium of *S. fimicola* stored large amounts of desthiobiotin whereas that of *C. pini* did not.

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# STUDIES ON THE MODE OF ACTION OF COMPOUNDS CONTAINING AVAILABLE CHLORINE

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In a study of the bactericidal properties of various forms of active chlorine, certain preliminary results have been obtained which concern the mechanism by which N-chloro compounds react in aqueous solution. Information of this type is valuable because this form of active chlorine is frequently a useful means of application, and because in many cases where chlorine or hypochlorites are used there may be present nitrogen compounds capable of forming N-chloro derivatives. The extent to which this occurs, and the nature of the resulting compound, will largely determine the result obtained.

In aqueous solution it is necessary to consider that the reactivity of the N-chloro compound may be due in whole or in part to the hypochlorous acid and hypochlorite ion that are present in accordance with the hydrolysis and ionization equilibria. Although the degree of hydrolysis may be very slight in many cases, its effect can be significant if the activity of hypochlorous acid is sufficiently great compared to the unhydrolyzed N-chloro molecule. The experimental results indicate, in a few representative cases, under what conditions and to what extent the bactericidal properties of N-chloro compounds can be attributed to the small concentrations of hypochlorous acid that may be present under equilibrium conditions.

It is assumed that the speed of hydrolysis is greater than the rate at which hypochlorous acid is consumed in killing the organisms and therefore need not be considered. The experimental conditions have been limited to dilute aqueous solutions, the pH values of which range from 4 to 9. With these restrictions, active chlorine in the form of dissolved molecular chlorine need not be considered, since its concentration would be insignificant (Jakowkin, 1899).

## EXPERIMENTAL PROCEDURE

**Organisms.** The spores of *Bacillus metiens* (Rudolph and Levine, 1941) were grown on agar flats at 37 C for one week at which time most of the organisms were in the spore stage. They were suspended in saline solution, and the suspension was washed twice by centrifuging and stored in the refrigerator until used. The *Escherichia coli* cells were grown in nutrient broth, removed by centrifuging, washed twice, and used immediately.

**Reagents.** Water, free from reducing properties, was prepared by adding 10 ppm chlorine to distilled water and permitting it to stand for about a week. It was then set in the sun for a few days to remove the active chlorine. Before use, it was checked to make sure that it had neither chlorine residual nor chlorine

demand. It was buffered with M/50 phosphate for pH studies in the range of pH 6 to 8, with acetate for studies below pH 6 and borate for studies above pH 8.

The 3,5,5-trimethylhydantoin was prepared by the method of Biltz and Slotta (1926) and recrystallized to constant biological activity.

N-methyl-*p*-toluenesulfonamide was prepared by the reaction of *p*-toluenesulfonyl chloride on methylamine (Crossley *et al.*, 1940).

Piperidine was obtained from the Eastman Kodak Company.

*Method.* Stock hypochlorite solutions were prepared from chlorine gas bubbled into sodium hydroxide solutions. The proper quantity was added to the buffered water or to the solution of the nitrogen compound in the buffer. Samples were removed initially and at times during the experiment, to determine the chlorine residuals. Available chlorine was determined by iodometric titration, and free chlorine was determined by amperometric titration (Marks and Glass, 1942). About 200,000 organisms per ml were added to the equilibrated solutions, and at measured time intervals aliquots were removed to tubes containing sufficient sterile sodium sulfite to inactivate the chlorine. Plate counts were made from these aliquots, and the logarithms of the percentage of survivors were plotted against time. The time for 99 per cent killing was estimated from these curves.

#### RESULTS

*Bactericidal properties of hypochlorous acid.* Before discussing the N-chloro compounds, it will be worthwhile to consider the properties of hypochlorous acid, in view of its apparent importance. Usually pH has a pronounced effect on the activity of available chlorine in any form and the nature of this effect should provide valuable information of fundamental significance. Weber and Levine (1944) have studied the influence of pH on the action of hypochlorous acid on *B. metiens* spores and this work has been expanded somewhat. The relation between pH and the rate at which hypochlorous acid kills spores has been studied over the pH range 6 to 9 with hypochlorous acid concentrations ranging from 0.5 to 450 ppm available chlorine. The concentration range changed as the pH changed in order to obtain 99 per cent killing in convenient and readily measurable time periods. In agreement with Levine it was found that at each pH value, the log of the time required for 99 per cent killing plotted against the log of concentration gave a straight line and that the rate of sterilization decreased rapidly as the pH increased.

Calculation shows that the variation of death rate with pH is such that it can be accounted for by assuming that undissociated hypochlorous acid is the active form and that hypochlorite ion is entirely inactive (Holwerda, 1928). This is illustrated in figure 1 in which the log of the time required for 99 per cent killing is plotted against the log of the concentration of undissociated hypochlorous acid as calculated from the total available chlorine concentration at each pH value. In the calculation of the concentration of undissociated hypochlorous acid, the value of  $6.8 \times 10^{-8}$  has been used as the ionization constant

(Holst, 1940). It will be noted that over the pH and concentration ranges used, all the points fall on the same straight line within a reasonable degree of error. Thus, the effect of pH on the sporicidal activity of active chlorine solutions of this type can be attributed to its effect on the relative amounts of hypochlorous acid and hypochlorite ion present. Such a result makes it reasonable to expect that the effect of pH on the activities of N-chloro compounds, for example, can be expressed by a combination of this relationship with one giving the pH dependence of other important factors, such as extent of hydrolysis.

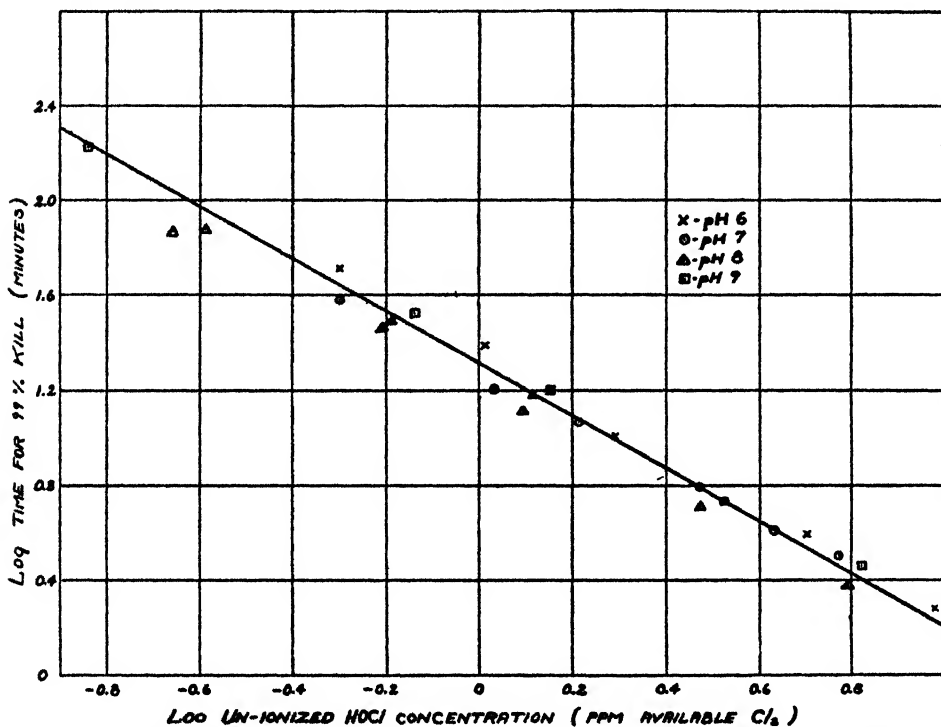


FIG. 1. SPORICIDAL ACTION AS A FUNCTION OF CONCENTRATION OF UN-IONIZED HYPOCHLOROUS ACID

*The activity of the unhydrolyzed molecule.* A factor other than the hydrolysis which may influence the reactivity of N-chloro compounds is the inherent toxicity of the unhydrolyzed molecule. For the purpose of getting an indication of the relative importance of the two forms, nitrogen compounds were chosen for illustrative rather than practical value. It is necessary to choose compounds and conditions such that the solutions are sufficiently stable in order that differences in killing rates cannot be attributed to different rates of loss of available chlorine. This is important in the studies with spores because of the long time periods that may be involved. The compounds should also be capable of forming only monochloro derivatives under the conditions of the experiment, so that changes in activity resulting from changes in nitrogen to chlorine ratio

cannot be attributed to changes in the relative proportions of monochloro and dichloro compounds.

In the following tables are included data on the killing rate as a function of the ratio of nitrogen to chlorine. Table 1 shows the effect on *B. metiens* spores of available chlorine in the presence of N-methyl-*p*-toluenesulfonamide, 3,5,5-trimethylhydantoin, and piperidine. Solutions of the first two compounds are sufficiently stable and the duration of the experiment is sufficiently short that practically no loss in chlorine occurs. For this reason the data obtained with these compounds are reproducible. Piperidine is included chiefly for the purpose of illustrating the large variations in activity that are encountered among N-chloro compounds; this one being relatively inactive. The long duration of the experiment and the high concentration required cause some losses in chlorine to occur, so that only the order of magnitude is important. Further, when the stoichiometrical quantities of nitrogen and chlorine are present, the

TABLE 1  
*Effect of nitrogen-chlorine ratio (N/Cl) on sporicidal activity*  
pH = 7; temp 25°C

N-METHYL- <i>p</i> -TOLUENESULFONAMIDE 25 PPM AVAIL. Cl <sub>2</sub>		3,5,5-TRIMETHYLHYDANTOIN 500 PPM AVAIL. Cl <sub>2</sub>		PIPERIDINE 400 PPM AVAIL. Cl <sub>2</sub>	
N/Cl	KT	N/Cl	KT	N/Cl	KT
1.2	0.90	1	0.22	2.0	9.0
1.4	1.13	3.0	1.03	2.5	14.0
1.6	1.25	4.0	1.13	10	22.0
1.8	1.35	5.0	1.58	20	24.0
2.0	1.40	7.0	1.57	40	26.0
5.0	1.40	10	1.63		
		20	1.63		

KT = Time in hours for 99 per cent kill.

sporicidal activity resides entirely in the small concentration of hypochlorous acid present, so that results obtained in such solutions are subject to considerable error.

It is observed that as the nitrogen is increased above that stoichiometrically necessary to form the N-chloro compound, the activity decreases in such a way as to approach a limit. It is evident that the decrease in activity corresponds to a decrease in the concentration of hypochlorous acid, owing to the displacement of the hydrolysis equilibrium. Eventually the point is reached beyond which further increase in the nitrogen concentration produces no significant change in activity. This is interpreted to mean that the concentration of hypochlorous acid has become so small that its activity is negligible with respect to that of the unhydrolyzed N-chloro compound. The bactericidal efficiency of the latter species is probably measured by the activity observed under these conditions.

Table 2 shows the effect of increasing concentrations of the three compounds

on the rate at which chlorine kills *Escherichia coli*. With this nonsporeforming organism, the bactericidal activity varies with nitrogen concentration in a fashion similar to that observed in the spore experiments. The activities of the three unhydrolyzed N-chloro compounds fall into the same order with *E. coli* as with *B. metiens* spores, although the resistance ratios are probably very different.

Although the toxicity of the unhydrolyzed molecules may vary widely, it is apparent that the bactericidal action of solutions of pure N-chloro compounds with no excess nitrogen will be largely dependent on the amount of hypochlorous acid present and hence on the hydrolysis constant. Attempts to measure this quantity have been only partially successful. Soper (1924) used a solubility method on the N-chloro-*p*-toluenesulfonamides and obtained reasonable values. However, even slight decomposition causes large errors in the method, so that it is of limited applicability.

TABLE 2

*Effect of nitrogen-chlorine ratio on death rate of Escherichia coli*  
pH = 7; temp 25 C; 2 ppm available chlorine

N/Cl	TIME FOR 99% KILL		
	N-methyl- <i>p</i> -toluenesulfonamide	3,5,5-trimethylhydantoin	Piperidine
<i>mols</i>	<i>min</i>	<i>min</i>	<i>min</i>
1	0.3		
10	1.1		170
20	1.2	1.3	260
30		2.3	
40	1.3	3.0	290
50		3.6	
60		3.8	
80	1.3	3.8	300
160		4.0	
320		3.9	

The data obtained with N-chloro-N-methyl-*p*-toluenesulfonamide are sufficiently reliable and complete to calculate an approximate value for the hydrolysis constant.

Figure 2 shows that the sporicidal activity of the N-chloro-N-methyl-*p*-toluenesulfonamide molecule varies with concentration in the same manner as that of hypochlorous acid. At each concentration the molar ratio of nitrogen to chlorine is five to one, which has been shown to make the hydrolysis negligible. From the relative activities of hypochlorous acid and the chloramide molecule over the proper concentration range, the hydrolysis constant is calculated to be about  $10^{-8}$ . For the first chlorine of the very similar dichloramine-T, Soper found  $8 \times 10^{-7}$ .

*Application to dichloramine-T and halazone.* An interesting application of the above results is found in the action of dichloramine-T and halazone on *B. metiens* spores as a function of pH. The results of such a study, carried out in the manner described, are given in table 3.

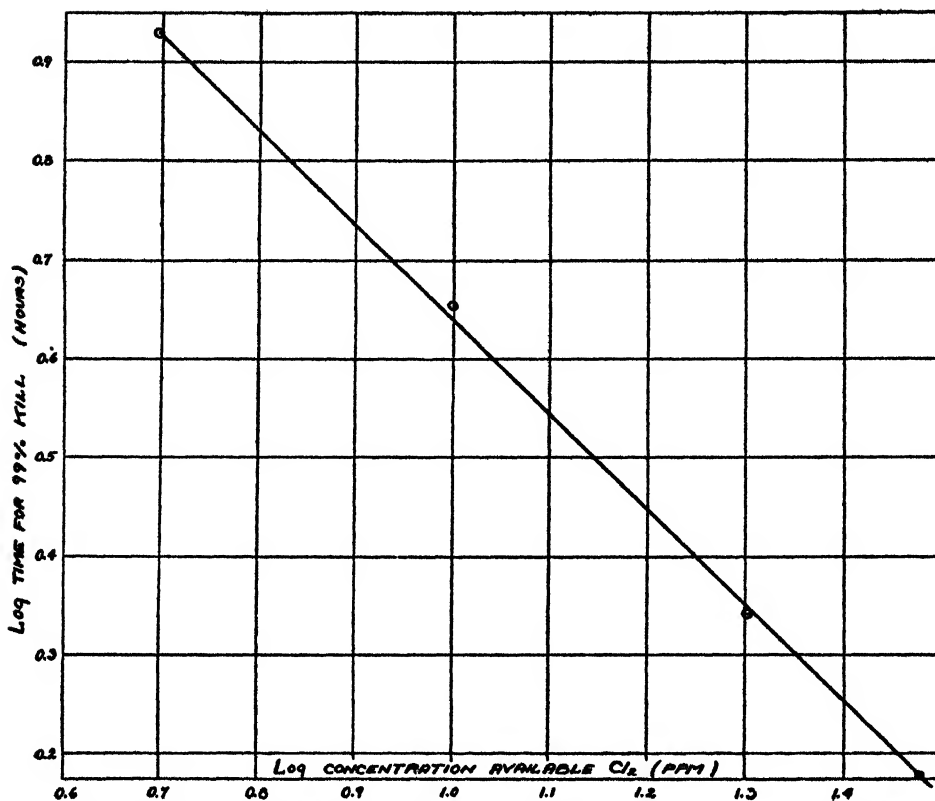


FIG. 2. SPORICIDAL ACTIVITY AS A FUNCTION OF THE N-CHLORO-N-METHYL-*p*-TOLUENE-SULFONAMIDE CONCENTRATION

Nitrogen to Chlorine Ratio = 5

TABLE 3

*Effect of pH on the sporicidal activity of dichloramine-T and halazone*

Temp 25 C; 5 ppm available chlorine

pH	TIME FOR 99% KILL	
	Dichloramine-T	Halazone
	min	min
3	87	35
4	72	33
5	60	26
6	24	13
7	16	9
8	42	43
9		60

The work of Soper on the extent of hydrolysis of such compounds shows that as the pH is increased the hydrolysis equilibrium is displaced further to the right (since both right-hand members are acids). At the same time this shift

in pH increases the extent of ionization of hypochlorous acid, this increase being most pronounced between pH 6.5 and 8.5. The increase in killing rate, as the pH is increased up to 7, can be attributed to an increase in hypochlorous acid concentration as a result of the displacement of the hydrolysis equilibrium. On the other hand, as the pH is increased above 7, the most important change becomes the increased ionization of hypochlorous acid resulting in decreased bactericidal activity.

#### SUMMARY

N-chloro compounds can act on microorganisms by at least two different mechanisms: (1) the molecule of the chlorine compound can act directly; (2) hypochlorous acid formed by hydrolysis may be responsible for the result. The latter substance is more reactive than the intact N-chloro compound, and, unless conditions are such as to make its concentration negligible, it will be a major factor in the rate of sterilization. This is one reason why pH is such an important factor in the application of compounds of this type. The pH of the solution determines the fraction of the hypochlorous acid in the un-ionized form to which the activity may be attributed. Since most nitrogen compounds which are capable of forming N-chloro derivatives are either acids or bases, the extent of hydrolysis will vary with pH even in that region where the hypochlorous acid remains substantially un-ionized. When the amine or amide nitrogen concentration becomes sufficiently great, the rate of sterilization depends on other properties of the chlorine compound. These properties appear to vary within wide limits among different compounds, so that in order to know what bactericidal activity to expect, it is necessary to know, not only the nitrogen to chlorine ratio, but also the nature of the nitrogen compound.

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# A STUDY OF CULTURAL METHODS FOR THE QUANTITATIVE DETERMINATION OF BACTERIAL POPULATIONS OF DISTILLERY MASHES<sup>1</sup>

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Quantitative cultural data on bacterial contamination in a distillery are difficult to obtain because ordinary methods will not distinguish between yeast and bacteria. Direct counts are unreliable because of the large number of grain particles which are difficult to distinguish from bacteria. This investigation was begun, therefore, in order to find a reliable cultural method for counting bacteria in distillery mashes.

A procedure which seemed worthy of investigation was the "tube count" method which has been used for many years by the Bureau of Dairy Industry of the United States Department of Agriculture in counting lactate-fermenting bacteria in Swiss cheese. Dr. W. C. Frazier of the University of Wisconsin suggested that the method had possibilities in routine work, and under his direction it was used extensively in research work on brick cheese (Garey *et al.*, 1941).

## METHODS

Of several special culture media tried, the following medium was found to give satisfactory results:

Tomato juice	400 ml
Difco yeast extract	10 g
Glucose	10 g
Salt solution A	5 ml
Salt solution B	5 ml
Distilled water	600 ml
Sodium hydroxide to pH	7.0 $\pm$ 0.1
Agar	15 g

The tomato juice was prepared by mixing approximately 1 g of filter cel with each 100 ml of commercially canned tomatoes and filtering through coarse paper by suction. Salt solution A contained 25 g each of mono- and dibasic potassium phosphate per 250 ml of distilled water. Salt solution B contained 10 g of magnesium sulfate heptahydrate, and 0.5 g each of sodium chloride, ferrous sulfate heptahydrate, and manganous sulfate tetrahydrate per 250 ml of distilled water. The medium was dispensed in approximately 10 ml amounts

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into 18 x 150 ml pyrex culture tubes which were stoppered with cotton and autoclaved for 30 minutes at 15 pounds.

When plain nutrient agar plate counts were used, standard procedures were followed (American Public Health Association, 1941).

"Sealing" agar was prepared as a 3 per cent agar-in-water solution and sterilized by autoclaving for 30 minutes at 15 pounds.

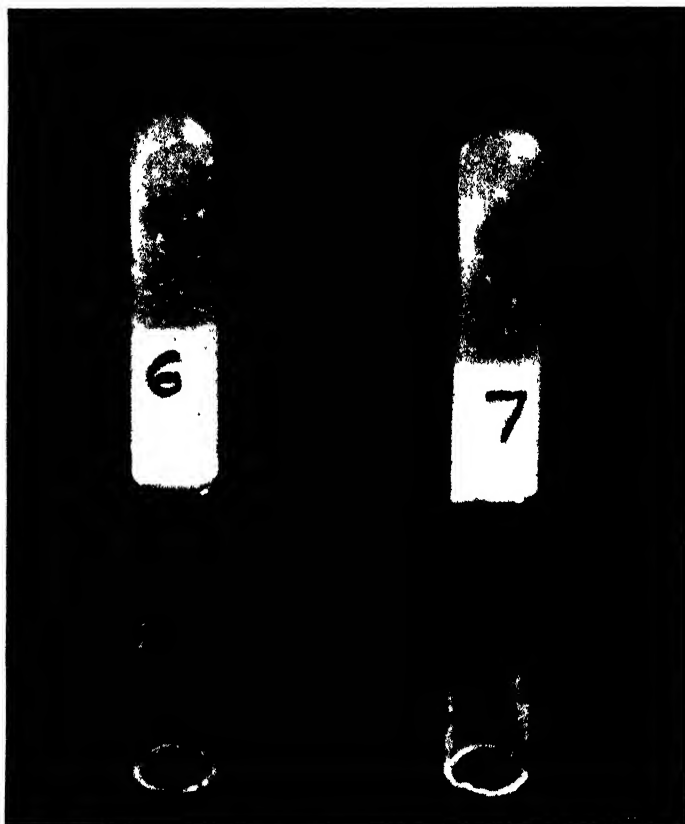


FIG. 1. THE APPEARANCE OF BACTERIAL COLONIES IN THE TUBE COUNT METHOD WHEN TOMATO JUICE AGAR AND A 3 PER CENT AGAR SEAL WERE USED. ("6" REFERS TO A 1:1,000,000 DILUTION; "7" TO A 1:10,000,000 DILUTION.)

Isolations were made from the "tube count" preparations by blowing the medium into a sterilized petri dish by means of 4-mm glass tubing which had been sterilized by dipping in 95 per cent alcohol and burning off the alcohol. Isolations from colonies could then be made easily by use of a stiff inoculating needle.

The tube counts were conducted by adding appropriate dilutions of the mash to tubes of melted and cooled (45 to 50 C) tomato juice agar, mixing, and allowing the agar to solidify. Approximately 5 ml of "sealing" agar were added to inhibit growth of yeasts and prevent "blowing" when gas-forming bacteria were present. Incubation was at 37 C for 24 to 48 hours.

## RESULTS

Figure 1 shows the appearance of bacterial colonies in tubes of tomato juice agar after 48 hours of incubation at 37 C. Up to 100 colonies per tube can be counted with a reproducibility of 5 per cent. When counting the colonies, it

TABLE 1

*Comparison of bacterial counts obtained on a fermenting grain mash by the tube count method with those found by the petri plate procedure*

TIME OF SAMPLING FERMENTER AFTER "SET"	NUMBER OF BACTERIA PER ML*	
	Tube count	Plate count
<i>hours</i>		
0	140,000	11,500
24	45,000,000	32,000
48	290,000,000	14,4000
72	400,000,000	1,100

\* It is of course recognized that the higher counts obtained with the tube count method may be due quite as much to the superior medium employed (as opposed to the nutrient agar used in the plate counts) as to the method itself. Experience has shown, however, that higher counts are frequently obtained with the tube technique when the same medium is used with the two methods.

TABLE 2

*Bacterial counts obtained by the tube count method on samples collected after malting, after cooling, and during fermentation of a grain mash*

	NUMBER OF BACTERIA PER ML	
	Before cleaning coolers	After cleaning coolers
First converted "cook" at cooker . . . . .	400	200
First cooled "cook" at fermenter. . . . .	140,000	200
Second converted "cook" at cooker. . . . .	300	100
Second cooled "cook" at fermenter. . . . .	14,000	<100*
Third converted "cook" at cooker. . . . .	<100	<100
Third cooled "cook" at fermenter. . . . .	<100	<100
Fourth converted "cook" at cooker. . . . .	100	100
Fourth cooled "cook" at fermenter. . . . .	4,000	200
"Set" fermenter. . . . .	40,000	3,000
"Dropped" fermenter. . . . .	400,000,000	180,000,000

\* All notations "less than 100" signify no colonies in tube of 1:100 dilution.

is desirable to use good illumination such as a reading lamp or a plate counter equipped with electric bulbs.

Table 1 demonstrates that the apparent bacterial content of distillery mash decreased in number during the fermentation when nutrient agar plate counts were used. When the same material was examined by the tube count method using tomato juice agar, it is seen that the numbers of bacteria increased steadily during the fermentation.

Table 2 shows one application of the tube count method to a plant contamina-

tion problem. Data on a "set" fermenter indicated that bacterial contamination was coming from the mash coolers. After a thorough cleaning of the coolers, the numbers of bacteria in the cooled mash decreased, and the "set" count was relatively low.

#### DISCUSSION

The tube count method failed to distinguish between yeast and bacteria unless the three per cent agar seal was used. The seal apparently prevents the diffusion of oxygen into the tomato juice agar and the yeasts will not develop into visible colonies under anaerobic conditions. When tube counts were made on yeast mash, the 1:100 and 1:1,000 dilutions would frequently show a haziness as a result of yeast growth, but no visible colonies developed unless bacterial contaminants were present; in higher dilutions the cloudiness due to yeast growth was never apparent. In "set" fermenters, colonies of yeast did not develop when the agar seal was used, even in a 1:100 dilution.

When extreme attention was paid to all details of technique, the results with the tube counts showed good correlation between dilutions. That is, when 1:1,000,000 tubes showed 100 colonies, 1:10,000,000 tubes would have 9 to 11 colonies. Plate counts never showed such good agreement.

#### SUMMARY

A cultural method for determining the bacterial content of distillery mashes is described. The method is easier to run than the plate count method, and the data are more reliable. The method can be used successfully for cultural counts of grains and water.

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## NOTES

### A MICROCOCCUS PRODUCING A MULBERRY-COLORED PIGMENT<sup>1</sup>

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Received for publication December 4, 1944

In the course of a study of contaminated wounds a pigment-forming micrococcus was isolated which differs from any adequately described in the literature. The known varieties of micrococci which form pigment produce a yellow, orange, or red color. The species which we isolated produced under certain conditions a mulberry-colored growth.

The characteristics of this organism are:

Morphology—Spheres, 0.9 micron in diameter, occurring in clusters; gram positive; nonmotile.

Aerobe—Facultative anaerobe.

Optimum temperature—37 C.

Growth in broth—Turbid with a white ring and sediment.

Agar slant—Moderate growth, white, glistening.

Agar colonies—White, opaque.

Gelatin—No liquefaction.

Potato slant—Mulberry-colored growth. Pigment noticeable at twenty-four hours but increased at forty-eight hours. The color does not diffuse into the potato.

Human blood agar—Hemolysis.

Litmus milk—Acid and coagulation.

Hydrogen sulfide—Negative.

Nitrate reduction—Variable.

Indole—Negative.

Carbohydrate fermentations—Acid, no gas in glucose, lactose, or sucrose.

No acid or gas in mannitol, salicin, raffinose, maltose, or glycerol.

Nonpigmented variants were frequently produced following icebox storage.

Coagulase tests on the three strains which we isolated were negative. Two of the strains reduced nitrate, one did not.

Two organisms described by Schroeter in 1872 and named in an article by Cohn may have been similar to the coccus we have isolated (*Beitr. Biol. Pflanz.*, **2**, 127; 190). Schroeter exposed slices of cooked potato to the air, and among the cocci which he isolated by this method was one, *Micrococcus cyaneus*, which produced a blue pigment and another, *Micrococcus violaceus*, which produced

<sup>1</sup> The work described in this report was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

a lavender growth. Agar had not yet been incorporated into culture media, and Schroeter could only describe the growth of these micrococci on potato. We are indebted to Eleanor H. Clise for her review of the literature covering the genus *Micrococcus*.

Among the micrococci this organism is unusual in forming pigment on potato, but none on agar medium. A marked difference in pigment production on potato compared to agar is well established for members of other genera, including *Pseudomonas*, *Erwinia*, *Serratia*, *Cellulomonas*, *Bacillus*, and *Clostridium*.

In case this organism should prove to be a species not heretofore described, we suggest the name *Micrococcus moricolor* from the Latin, "color of mulberry."

The organism described in this paper was isolated three times in a series of 140 micrococci and staphylococci. The coccus was recovered from the open wounds of three different patients, where it apparently occurred as a contaminant.

## EFFECT OF INTRACRANIAL PENICILLIN THERAPY ON BRAIN INVOLVEMENT IN EXPERIMENTAL RELAPSING FEVER

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Received for publication February 3, 1945

In a previous paper (Science, **100**, 550) we reported failure to cure brain involvement in white rats infected with relapsing fever spirochetes (strain transmitted by *Ornithodoros turicata*) with intraperitoneal injections of penicillin adequate to prevent brain involvement and to cure blood stream involvement. We now have resorted to combined intracranial and intraperitoneal injections of the penicillin.

Seventeen rats which had been infected 12 to 40 days previously were divided into 6 groups. Groups I, II, and III were test groups of 3 rats each which received 1,000 units of penicillin intracranially, in addition to intraperitoneal injections of 400 units every 3 hours for 48 hours. Group I rats received 1,000 units of penicillin in a single intracranial injection. Group II rats received 2 intracranial injections of 500 units each, and group III rats received 3 intracranial injections of 333 units each at intervals of 3 hours. Group IV consisted of 3 control rats which received intracranial injections of buffer solution (penicillin diluent) comparable in amounts to the penicillin received by the test rats in groups I, II, and III. Group IV rats were anaesthetized with the same dose of phenobarbital used on the test rats, and received the same total dose of penicillin (7,800 units) as the test animals in 17 intraperitoneal injections. Group V consisted of 2 control rats which received no phenobarbital and no intracranial injections, but received the same total amount of penicillin (7,800 units) as the test animals in 17 intraperitoneal injections. Group VI consisted of 3 control rats which were untreated.

One rat in group I, one in group III, and two in group IV died within a few hours after the first intracranial injection and were discarded. One additional rat in each of the test groups I, II, and III died during the course of intraperitoneal injections after receiving 1,600, 2,000, and 5,200 units of penicillin, respectively, in addition to 1,000 units intracranially. These rats were placed in the icebox and were used in the brain passage phase of the experiment. The death of these animals was attributed to barbiturate poisoning, although brain injury may have been involved, especially in the early deaths.

Two days after termination of the penicillin treatment, the brains of the 4 surviving, the 3 icebox-preserved test rats, and the 6 surviving control rats were removed, emulsified, and passed to fresh rats. Uniform (0.01 ml of 1:20 dil.) dark-field preparations of the tail blood of each of these passage rats were examined daily for 10 days. All 6 of the control passage animals became positive within 3 to 6 days, whereas the 7 test passage animals remained negative throughout the ten-day examination period.

Thus it would appear that penicillin injected intracranially is capable of curing the brain involvement in experimental relapsing fever of the white rat. Minimum curative doses for both brain and blood stream involvement remain to be determined.

## INCREASING PENICILLIN YIELDS WITH CORN OIL

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Investigative work on antibiotic agents is proceeding so rapidly the lone investigator often finds that what he considers a fundamental discovery is a recognized fact among research groups engaged in these studies. However, the writer has made an observation while studying the growth habits and nutritive requirements of *Penicillium notatum* that apparently has not been recorded in the literature.

In an effort to find an agent that would readily float the mold spores on the surface of a fluid medium, corn oil was added in 2 per cent concentration to Czapek-Dox medium modified by the substitution of brown sugar for glucose. In due time it became evident that the corn oil served as more than a mechanism for floating the spores and permitting the ready development of an abundant and uniformly distributed mold growth. After an initial drop in acidity, the pH of the broth returned to 6.9, where it remained relatively constant for the duration of the experiment. The penicillin content of the medium gradually increased and on the twenty-second day after seeding was sufficiently concentrated to permit a 1:320 dilution of the broth to induce inhibitory zones with diameters of 22 to 25 mm on agar plates inoculated with the "H" strain of *Staphylococcus aureus*. Eimer and Amend "penicylinders" were used as test



cups. After the twenty-second day, the penicillin content of the corn oil medium began to decrease and had disappeared by the thirtieth day.

The addition of lactose to the brown sugar corn oil medium did not increase penicillin formation by the mold, but its presence gave evidence of exerting a preservative or stabilizing effect on the antibiotic. Penicylinders filled with 1:320 dilutions of this medium were surrounded by inhibitory zones measuring 20 to 26 mm in diameter on plate cultures of *Staphylococcus aureus* after 30 days of incubation. Lower dilutions, for example the 1:10, produced zones of inhibition measuring as much as 40 mm in diameter.

Two other media were employed with the corn oil preparations. One consisted of the Czapek-Dox brown sugar modification alone, and the second incorporated lactose without corn oil. The penicillin concentration of the brown sugar medium was ineffective beyond a 1:5 dilution, and exerted its antibiotic effect only between the seventh and ninth day of incubation, after which the broth shifted rapidly to an alkaline state. The brown sugar medium incorporating lactose without corn oil produced inhibitory zones 22 to 26 mm in diameter on *Staphylococcus* plate cultures in dilutions of as much as 1:20 from the ninth to the eighteenth day of incubation. The inhibitory effect disappeared after the twentieth day.

All media were employed simultaneously, in like amounts and in containers providing equal surface areas. Each broth was seeded with 1 ml of spore suspension and incubation was conducted at 22 C.

Whether the corn oil tends to adsorb the penicillin as formed and prevent its rapid disintegration and loss from the medium, whether it tends merely to stabilize the pH, whether it provides an ideal carbon source enhancing penicillin production by the mold, or contributes to a combination of these factors, are subjects for further investigation.

# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## NORTHERN CALIFORNIA-HAWAIIAN BRANCH

STANFORD UNIVERSITY, CALIFORNIA, JULY 29, 1944

**YEAST PRODUCTION.** *E. C. Saudek*, Consumer's Yeast Company, San Francisco.

Yeast production and its problems involve many phenomena. Particular consideration is given to raw materials, process, product, and research. The adoption of molasses as a raw material and the process that accompanies its use are the greatest factors leading to the production of the modern uniform yeast. The old method of using grain introduced variables, many of which could not be controlled or compensated.

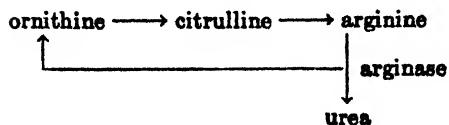
Careful and rigid application of bacteriology, biochemistry, and engineering have led to many improvements in sanitation, yeast culturing, yeast nutrition, and process control.

The results of research, including the necessary yeast growth factors, yeast breeding, and dry viable yeast, will soon create new revolutionary advances in yeast production and marketing.

### ARGININE METABOLISM IN *NEUROSPORA*.

*A. M. Srb*, Stanford University.

Studies with *Neurospora* confirm the ornithine cycle:



In irradiated *Neurospora* mutations have occurred to give rise to fifteen "arginineless" strains; in each of seven the mutant character is due to the effect of a different single gene. The strains fall into three groups. In group I (four mutants), growth takes place if either ornithine, citrulline, or arginine is supplied in addition to the minimal medium of wild type. In group II (two mutants), growth occurs if citrulline or arginine is provided. The one strain in group III has a specific requirement for arginine. This means that ornithine and citrulline represent different stages in the synthesis of arginine in *Neurospora*, and

occur in the order postulated in the Krebs-Henseleit cycle above.

The sequence of reactions is confirmed by the behavior of double mutant strains which were obtained by crossing different single mutants. In addition, both arginase and urease have been demonstrated in *Neurospora*.

If an arginineless strain of group I is grown on ornithine, and an autolysate of the resultant mycelium is prepared, the mutant with a specific requirement for arginine can grow on minimal medium plus the autolysate. This proves that arginine is actually produced in the strains grown upon ornithine.

### A LYSINE-REQUIRING MUTANT STRAIN OF *NEUROSPORA*.

*A. H. Doermann*, Stanford University.

Bioassays of lysine by means of "lysineless" mutant strains of *Neurospora* check closely with chemical assays. The objection to chemical assay methods is that all depend first on original isolation of protein and are very laborious.

Difficulties with the bioassay of lysine by means of *Neurospora* are, first, that arginine inhibits all lysineless mutants, and, second, that other amino acids variably stimulate these strains. The first problem has been solved by silver precipitation of the arginine. The variable stimulation has been answered by adding asparagine and glutamic acid to the media, for they achieve maximal stimulation and variability of other amino acids can have no effect.

After suitable acid hydrolysis and silver precipitation the material to be tested is added to basal media containing asparagine and glutamic acid. The lysineless strain of *Neurospora* is grown until the lysine is completely exhausted. The mycelium is then weighed.

The 168 hours necessary for this method of bioassay of lysine makes it cumbersome, but another approach is now being worked out which promises to be much more rapid.

**FOOD PRESERVATION.** *E. M. Mrak*, University of California.



# THE SO-CALLED GENUS CANDIDA BERKHOUT, 1923

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## I. INTRODUCTION

The well-known researches of Robin (1853), Quinquaud (1868), Hansen (1888), Vuillemin (1911), Castellani (1911, 1913, 1914, 1937), Berkhout (1923), Ciferri and Redaelli (1929), Stovall and Bulbolz (1932a, 1932b), Benham (1931), and Langeron and Talice (1932) mark the principal stages in the development of knowledge regarding the nonascosporogenous and filamentous yeasts called "monilias."

During the past years several new papers have been published. They may be classified in two groups in accordance with the procedures used for the identification of the species within the genus *Candida*. Martin *et al.* (1937) and Martin and Jones (1940) adopted the method summarized by Conant (1940), whose procedures are as follows: (a) appearance of the growth in Sabouraud's glucose broth, on Sabouraud's glucose agar, and on beef-extract blood agar plates at pH 7.4 is studied; (b) the microscopical details are studied on corn meal agar slide cultures; (c) the fermentation reactions are tested in beef extract broth with glucose, maltose, sucrose, and lactose, in tubes sealed with sterile vaseline, and incubated at 37 C for 10 days. Conant recognized 7 species, *Monilia albicans*, *M. parakrusei*, *M. krusei*, *M. tropicalis*, *M. pseudotropicalis*, *M. stellatoidea*, and *M. guilliermondi*. On the other hand, Langeron and Guerra (1938) adopted more complex methods in accordance with the procedures that Stelling-Dekker (1931) and Lodder (1934) used for the study of the ascosporogenous and non-ascosporogenous yeasts. They studied the appearance of the growth on Sabouraud's agar, in 2 per cent glucose, 1 per cent peptone broth, and in Raulin's medium; the morphology was studied on slide cultures. The study of the biological characteristics included: (a) fermentation reactions with glucose, sucrose, lactose, maltose, fructose, galactose, and raffinose in tubes sealed with paraffin; (b) the study of the assimilation of these carbohydrates and of nitrogen (peptone, ammonium sulphate, asparagine, urea, histidine, glycoll, potassium nitrate, and sometimes tryptophane); (c) utilization of ethyl alcohol; (d) growth in milk; (e) liquefaction of gelatin. Langeron and Guerra accepted 16 species which include those accepted by the American authors except *Monilia stellatoidea*, which they did not study.

Conant does not admit as valid some species admitted by Langeron and Guerra: *Candida triadis* and *C. aldoi* of the classification of Langeron and Guerra are considered by Conant as synonyms of *Candida* (*Monilia*) *albicans*; *Candida intermedia* as identical with *Candida tropicalis*; *Candida brumpti*, *Candida chalmersi*, and *Candida flareri* as synonyms of *Candida parakrusei*.

Diddens and Lodder (1939a) recognized the genera *Candida* Berkhout and

*Trichosporon* Behrend in the subfamily *Mycotoruloideae* and, in a very short paper (1940), they announced that they admitted 23 species in the genus *Candida*.

TABLE 1

*Differential biological properties for the species of the genus Candida Berkhout*

	ZIMOGRAM				AUXANOGRAM WITH SUGARS						AUXANOGRAM WITH NITROGENOUS COMPOUNDS					OTHER PROPERTIES			
	Glucose	Sucrose	Lactose	Maltose	Glucose	Sucrose	Lactose	Maltose	Galactose	Raffinose	Inulin	Urea	Potassium nitrate	Ammonium sulphate	Asparagine	Peptone	Optimal temperature (high or low) in C	Experimental virulence degree for rabbits	Utilization of ethyl alcohol
<i>C. albicans</i> ....	G	-	-	G	+	+	-	+	+	-	-	-	-	+	+	+	30-37	1°	-
<i>C. stellatoidea</i> ....	G	-	-	G	+	-	-	+	+	-	-	-	-	+	+	+	30-37	-	-
<i>C. tropicalis</i> ...	G	G	-	G	+	+	-	+	+	+	-	-	-	+	+	+	30-37	2°	-
<i>C. intermedia</i> ..	G	G	-	G	+	+	+	+	+	+	-	-	-	+	+	+	20-30	-	+
<i>C. pelliculosa</i> ..	G	G	-	G	+	+	-	+	+	-	-	-	-	-	+	+	30-37	-	-
<i>C. krusei</i> *...	G	-	-	-	+	-	-	±	±	-	-	+	-	+	+	+	30-37	-	+
<i>C. parakrusei</i> ..	G	-	-	-	+	+	-	+	+	-	-	-	-	+	+	+	30-37	-	-
<i>C. guilliermondi</i> ....	G	G	-	-	+	+	-	+	+	+	-	-	-	+	+	+	30-37	-	-
<i>C. chalmersi</i> ...	G	G	-	-	+	+	-	+	+	-	-	-	-	+	+	+	30-37	-	-
<i>C. macedoniensis</i> ....	G	G	-	-	+	+	-	-	+	+	+	-	-	+	+	+	30-37	-	-
<i>C. pseudo-tropicalis</i> ....	G	G	G	-	+	+	+	-	+	+	+	+	-	+	+	+	30-37	-	-
<i>C. brumpti</i> ....	-	-	-	-	+	-	-	+	+	-	-	-	-	+	+	+	20-30	-	+
<i>C. flareri</i> †....	-	-	-	-	+	+	-	+	+	+	-	or +	-	+	+	+	20-30	-	-
<i>C. suaveolens</i> ..	-	-	-	-	+	+	+	+	+	-	-	+	-	+	+	+	30-37	-	+
<i>C. deformans</i> ‡	-	-	-	-	+	-	-	-	+	-	-	+	-	+	+	+			+
<i>C. zeylanoides</i> ‡	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+			-

\* The auxanogram with sugars was studied in Laurent's liquid medium.

† The auxanogram with the urea is positive or negative depending upon whether or not yeast or yeast extract is added to the medium.

‡ These strains were not personally studied and the data recorded are taken from Langeron and Guerra.

*dida*. We cannot offer an opinion about Diddens and Lodder's species until their descriptions are published in detail.

We studied 14 of the 16 species of Langeron and Guerra and also several strains of *Candida stellatoidea*, using the methods of those authors. However,

the assimilation of nitrogen was studied only with peptone, asparagine, urea, ammonium sulphate, and potassium nitrate, which we consider sufficient for the identification of the "monilias." The auxanographic procedure of Beijerinck was found efficient for the study of the assimilation of nitrogen and carbohydrates by almost all the species, but sometimes it failed to show clear results; in these cases we used the accessory procedures detailed in the following section. We also studied the dissociation (variation) and the experimental virulence of each species.

There exist several differences between our results and those of Langeron and Guerra, even when the same strains were studied and the same procedures used; we shall endeavor to explain the causes of these differences. Neverthe-

TABLE 2

LANGERON AND GUERRA'S CLASSIFICATION	CONANT AND DUKE UNIVERSITY'S CLASSIFICATION	ACTUAL CLASSIFICATION
<i>Candida albicans</i>	<i>Monilia albicans</i>	<i>Candida albicans</i>
<i>Candida triadis</i>	<i>Monilia albicans</i>	<i>Candida albicans</i>
	<i>Monilia stellatoidea</i>	<i>Candida stellatoidea</i>
<i>Candida tropicalis</i>	<i>Monilia tropicalis</i>	<i>Candida tropicalis</i>
<i>Candida intermedia</i>	<i>Monilia tropicalis</i>	<i>Candida intermedia</i>
<i>Candida pelliculosa</i>		<i>Candida pelliculosa</i>
<i>Candida pseudotropicalis</i>	<i>Monilia pseudotropicalis</i>	<i>C. pseudotropicalis</i>
<i>Candida guilliermondi</i>	<i>Monilia guilliermondi</i>	<i>C. guilliermondi</i>
<i>Candida chalmersi</i>	<i>Monilia parakrusei</i>	<i>Candida chalmersi</i>
		<i>C. macedoniensis</i>
<i>Candida krusei</i>	<i>Monilia krusei</i>	<i>C. krusei</i> , <i>Mycoderma cerevisiae</i> , and <i>Pichia krusei</i>
<i>Candida parakrusei</i>	<i>Monilia parakrusei</i>	<i>Candida parakrusei</i>
<i>Candida aldoi</i>	<i>Monilia albicans</i>	<i>Candida albicans</i>
<i>Candida brumpti</i>	<i>Monilia parakrusei</i>	<i>Candida brumpti</i>
<i>Candida flareri</i>	<i>Monilia parakrusei</i>	<i>Candida flareri</i>
<i>Candida zeylanoides</i>		Not studied
<i>Candida deformans</i>		Not studied
<i>Candida suaveolens</i>		<i>Candida suaveolens</i>

less, our conclusions are not unlike those of the French authors; we admit all their species except *Candida triadis* and *C. aldoi*, which are synonyms of *C. albicans* as stated by Conant, and we add to their list *Candida stellatoidea* and *C. macedoniensis*. Other species do not merit inclusion in the genus.

In table 1 we summarize the biochemical characteristics of the species admitted by us in accordance with our own experience. In table 2 we compare our conclusions with those of Langeron and Guerra, and those of Martin and Jones.

## II. METHODS

*Isolation and purification of cultures.* The cultures were purified by streaking on Sabouraud's agar plates.

*Optimal temperature.* This was determined by placing the cultures at three temperatures: 20 to 25 C, 25 to 30 C, and 37 C. This is a very important step in the study of a strain because the biological characteristics must be studied at the optimal temperature. *Candida brumpti* does not grow at 37 C, and other species grow badly.

*Appearance of growth.* It was studied in 2 per cent glucose, 1 per cent peptone broth and on Sabouraud's agar; sometimes also in beer wort and on beer wort agar.

*Morphology.* Slide cultures gave us, in general, good results. Two media were used: 2 per cent glucose, 1 per cent peptone soft (0.6%) agar and potato soft (0.6%) agar. The slides were inoculated and placed during 6 days in the incubator at 25 to 30 C in petri dishes, dried, then covered with a thin collodion film and afterwards colored with aqueous 0.2 per cent erythrosine, 1 per cent orange G, or with a 1 per cent cotton blue (CBBBB Poirrier) solution in Amman's lactophenol.

*Production of ascospores.* This was investigated on Gorodkova's agar, on potato and carrot slices, and on sterile blocks of plaster of Paris moistened with sterile saline. These cultures were placed at 15 to 20 C and were examined during two months in films dried in the air, then passed through a flame 3 or 4 times, and stained with carbol fuchsin at 100 C for five minutes, washed with water, destained with 2 per cent lactic acid alcohol for a few seconds, counter-stained with Nile blue and afterwards with India ink (*vide* Langeron and Guerra's paper).

*Growth in ethyl alcohol.* The medium recommended by Lodder was used (magnesium sulphate 0.05 g, potassium dihydrogen phosphate 0.1 g, ammonium sulphate 0.1 g, and distilled water 100 ml). Three per cent alcohol was added to one tube just before use, and nothing to the other, and the resultant growths were compared.

*Fermentation tests.* These were performed in 1 per cent peptone with 4 per cent of sugar, in tubes sealed with paraffin, at 25 to 30 C. The tubes were observed during 20 days.

*Assimilation of nitrogen.* The auxanographic procedure was used with the medium recommended by Lodder (glucose, 2 g; potassium dihydrogen phosphate, 0.1 g; magnesium sulphate, 0.05 g; agar, 2 g; distilled water, 100 ml). When the auxanographic method did not give clear results, we used Lodder's medium with 1 per cent of the nitrogen compound in tubes, and the growth on the surface was compared with that of a culture on Lodder's medium without any nitrogen compound.

*Assimilation of sugars.* The same auxanographic procedure was used almost always, but sometimes it failed to give good results. We then employed Laurent's liquid medium with 2 per cent of the sugar to be tested (ammonium sulphate, 4.71 g; potassium dihydrogen phosphate, 0.75 g; magnesium sulphate, 0.1 g; and distilled water, 1 L). The comparison of growths in this medium tested with and without the sugar indicates the capacity of a species to utilize the sugar.

*Experimental virulence.* We inoculated rabbits intravenously with 500 to 1,000 millions of blastospores of a culture on Sabouraud's glucose agar. A fresh culture of 24 to 48 hours must be used. If the rabbits died within 2 to 5 days after inoculation, with small and numerous abscesses of the kidneys ("systemic" or "disseminated lesions"), we said that the strain had a "first degree," or high, virulence (Mackinnon and Rodriguez-Garcia, 1935). If the rabbits did not die, we killed them on the tenth day after inoculation, and if they showed lesions of the tubes of the kidneys ("elimination lesions"), we said that the strain had a "second degree," or low virulence. If the rabbits sacrificed on the tenth day after inoculation did not show any lesion, and the cultures from the kidneys failed to show any growth, we said that the strain examined was not virulent.

*Action upon gelatin.* It was observed during two months.

### III. CHARACTERISTICS AND IDENTIFICATION OF THE SPECIES

#### 1. *Candida albicans* (Robin) Berkhout, 1923

*Cultures studied.* *Mycotorula albicans*, Langeron's strain 104. *Mycotoruloides triadis*, Langeron's strain 587. *Monilia aldoi*, Pereira's strain received from Langeron. *Monilia albicans*, phase "S," Negroni's strain. *Blastodendron erectum*, Langeron's strain 417. *Monilia pinoyi*, Castellani's strain. *Monilia psilosis*, Ashford's strain. *Candida albicans*, strain 582, Mackinnon. Also, 50 strains isolated in Montevideo from diverse origins.

The microscopic appearance of this species was thoroughly studied by Benham (1931), Langeron and Guerra (1938), Wickerham and Rettger (1939), Studt (1941), and others. A true mycelium and a pseudomycelium were observed as well as chlamydo-spores. The pseudomycelium showed amounts of blastospores at the distal end of each segment, resembling the mycotorula and mycotoruloides types of Langeron and Talice.

In table 1 we may observe that the auxanogram is negative with urea, which does not agree with Langeron and Guerra's results. Among the strains which we examined there were also several examined by Langeron and Guerra. We always used Merck's "extra pure" crystallized urea and new bacto agar. We have always obtained negative auxanograms with urea even after 12 days in the incubator. Nevertheless, when we prepared the medium with agar, reclaimed by the method of Thaller (1942), *Candida albicans* utilized the urea, producing clear positive auxanograms. The same phenomenon was observed with *Candida tropicalis* and *Candida parapsilosis*. These results seem to prove that some impurities of the urea or the agar, perhaps growth factors, are the cause of the differences between our results and those of Langeron and Guerra, and that very pure products must be used when the auxanogram tests are performed.

*Candida albicans* is a protean species, and through dissociation we have obtained two types of variants (Mackinnon, 1936, 1940a, 1940b): the "lethal variant" described by Negroni (1935) as an "R" form, and the "membranous



variant" described by us. Mackinnon (1936) stated from the very beginning of his experiments that his "membranous variant" was quite different from Negroni's variant; the "membranous variant" is homologous to the "R" form of bacteria and may be considered as a real "R" form. On the other hand, Negroni's variant cannot be considered as a true "R" form and may be related to the "lethal races" produced by Krassilnikov (1934) and Nadson (1937) from *Saccharomyces cerevisiae*.

The "lethal variant" is characterized by a slow rate of growth, by a great diminution or even a total loss of the experimental virulence, and by difficulty in production of mycelial growth. The cultures must be transferred every month, otherwise they die.

The "membranous variant" is characterized by the elongation of the blastospores into filaments (pseudofilaments), by the spiky and hard appearance of the cultures, by the production of a veil in liquid media, and by a diminution of the experimental virulence (2d degree).

Since between the normal form and the more accentuated (lethal and membranous) variants there exist intermediate variants, Mackinnon preferred to describe "directions of variation" rather than singular variations. Mackinnon also demonstrated that the membranous and the lethal variations may be produced in the same strain, which gives rise to variants which combine lethal and membranous characteristics ("lethal-membranous" or "lethal-R" variants).

We have not observed total or partial reversions from the lethal to the normal form although our observations have been extended over a period of 5 years. Partial reversions from the "R" to the normal or "S" form have been observed. The reverted forms, however, were unstable and tended to turn back again to the "R" form. Only once did we obtain a fixed "S"-like reversion, but the experimental virulence remained at a low degree (2d degree). Consequently, we have never observed a total reversion of the variants of *Candida albicans* after 5 to 7 years of observation, and both the "R" and the "lethal" variants are sufficiently stable to be considered true variants or mutants.

Langeron and Guerra (1939b) denied the real existence of dissociation. We think that the French authors have confused true dissociation or variation with nonhereditary changes of the appearance of the cultures on different media or on media the pH of which becomes alkaline. Langeron and Guerra cited the experiments performed by Teissier (1897), who obtained smooth growths on acid media, and membranous, rough cultures on alkaline media; we agree that this phenomenon cannot be considered as a dissociative variation or as a mutation because these smooth and rough appearances are not fixed and persist only as long as the media are acid or alkaline.

We have also observed that the capacity of *Candida albicans* to ferment maltose may be periodically lost. This phenomenon has been observed in three strains, including strain 606 named *Candida aldoi*. Martin and Jones (1940) observed irregularities in the ability of *C. aldoi* to ferment maltose, and Conant claimed that *C. aldoi* gave *C. albicans* fermentations after smooth colonies were isolated. Langeron and Guerra have written that *C. aldoi* is different from *C. albicans*

because it does not assimilate urea, but we have found that *C. albicans* also does not assimilate urea. *C. aldoi* produces chlamydospores. Consequently, in agreement with Conant we think that *C. aldoi* must be considered as a synonym of *C. albicans*. The culture which we have studied was in the "R" form and had a second degree experimental virulence.

*Candida triadis* (Langeron and Talice) is admitted as a good species by Langeron and Guerra, who, nevertheless, say that it is very similar to *C. albicans*. The French authors stated that *C. triadis* is a good species because it is more deeply yellowish than *C. albicans* and produces a mucous veil in liquid media, and because the blastospores are elongated. Moreover, it produces a partial veil in the ethyl alcohol medium; it has a second degree experimental virulence and produces typical chlamydospores similar to those of *C. albicans*. We think that *C. triadis* is a variant of *C. albicans*, intermediate between the "S" and the "R" forms.

## 2. *Candida stellatoidea* (Martin, Jones, Yao, and Lee) Langeron and Guerra, 1939

*Cultures studied.* *Monilia stellatoidea*, 2 strains received from N. Conant, and 3 from R. W. Benham, who received them from the Duke University School of Medicine.

This species must be admitted as a good one. It is different from *C. albicans* because the auxanogram is negative with sucrose, and because it is not virulent at all for rabbits.

We have obtained, through dissociation, "S" and "R" colonies, but they are not so easily differentiated as similar variants of *C. albicans*, because the "S" form of *C. stellatoidea* produces filaments very easily. On Sabouraud's agar, the "S" form produces a smoother growth than the "R" form (figures 1 and 2). The true mycelium is rather abundant even in the "S" form, and we have observed the production of appressorialike structures. The chlamydospores are similar to those of *C. albicans* (figure 3).

Langeron and Guerra (1939a) claimed that they had isolated a strain of *C. stellatoidea*, the auxanogram of this strain was positive with sucrose, but the experimental virulence was not studied. We think that Langeron and Guerra dealt with the "R" form of *C. albicans* and not with *C. stellatoidea*.

## 3. *Candida tropicalis* (Castellani) Berkhout, 1923

*Cultures studied.* *Monilia tropicalis*, Castellani's strain, Ross Institute. *Candida tropicalis*, Langeron's strain 255. *Candida tropicalis*, strain 360 isolated in Montevideo from paronychia. *Candida tropicalis*, strains 334 and 839 isolated in Montevideo from bronchomoniliasis. Four strains received from A. E. Arêa Leão and isolated in Brazil from bronchomoniliasis. Strain 267 of Langeron.

This species produces easily a true mycelium and a pseudomycelium with verticils of new blastospores. Single blastospores may also be seen attached to the sides of the hyphae; these blastospores were called pseudoconidia by Lan-

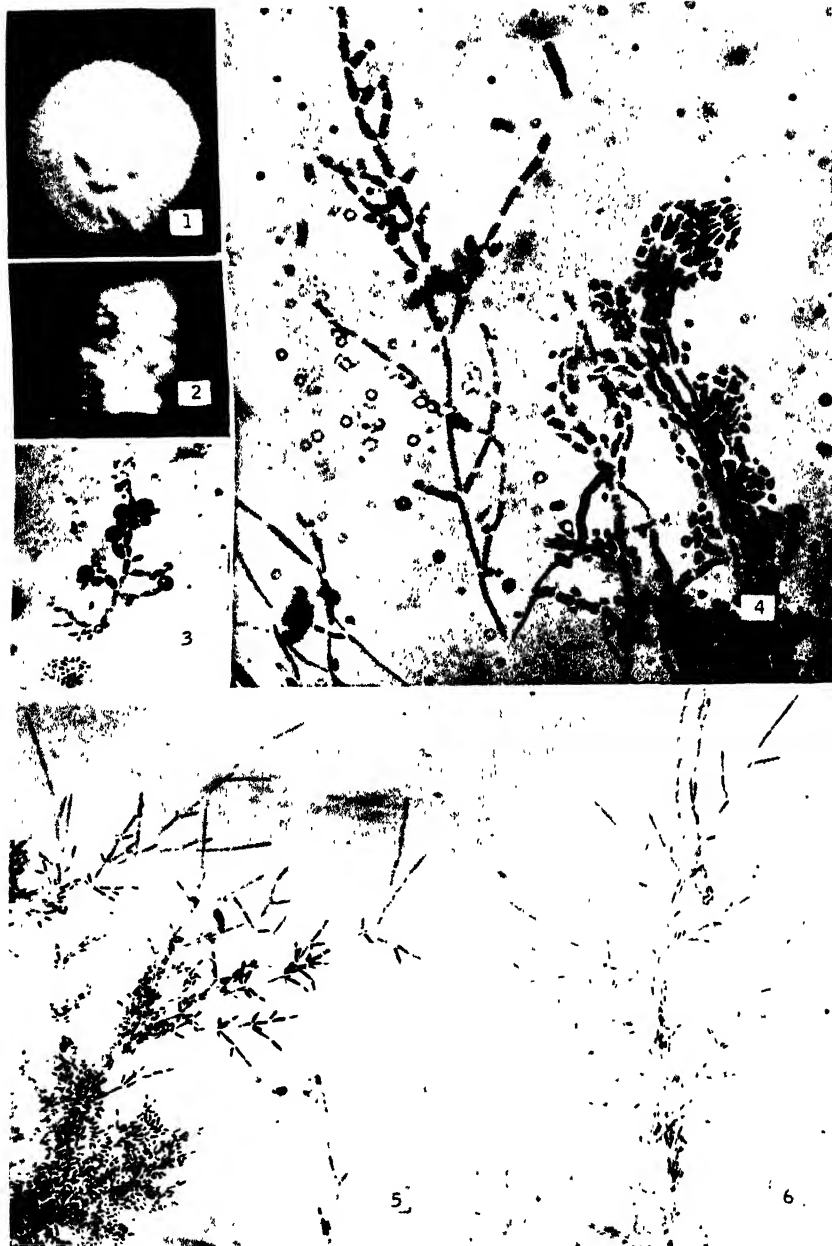


FIG. 1. *CANDIDA STELLATOIDEA*. "S" FORM. NATURAL SIZE ( $\times 1$ ). COLONY ON SABOURAUD'S GLUCOSE AGAR AGED 18 DAYS

FIG. 2. *CANDIDA STELLATOIDEA*. "R" FORM. NATURAL SIZE ( $\times 1$ ). COLONY ON SABOURAUD'S GLUCOSE AGAR AGED 18 DAYS

FIG. 3. *CANDIDA STELLATOIDEA*. SLIDE CULTURE. TYPICAL CHLAMYDOSPORES ( $\times 200$ ).

FIG. 4. *CANDIDA TROPICALIS*. STRAIN 360 APPRESSORIA AND FILAMENTS ON SLIDE CULTURES. MAGNIFICATION:  $\times 275$

FIG. 5. *MYCODERMA CEREVISIAE* (*CANDIDA KRUSEI*). TREELIKE STRUCTURES ON SLIDE CULTURES. STRAIN 774 ISOLATED FROM THE INDIAN BEER NAMED "CHICHA." MAGNIFICATION:  $\times 195$

FIG. 6. *MYCODERMA CEREVISIAE* (*CANDIDA KRUSEI*). STRAIN 575, ISOLATED FROM THE NORMAL SKIN. SLIDE CULTURE MAGNIFICATION:  $\times 175$

geron and Talice; sometimes we saw short chains of a few blastospores instead of single blastospores. Some strains produce more hyphae than others. True arthrospores and even appressoria may be seen (figure 4).

All strains showed a low experimental virulence (2d degree), and sometimes we had to inoculate several rabbits in order to observe various scarce and small lesions in the kidneys.

The auxanogram with urea is negative when new bacto agar is used in the preparation of the medium, but positive results were obtained when reclaimed agar was used.

#### 4. *Candida pelliculosa* Redaelli, 1925

*Culture studied.* Redaelli's strain received from Langeron. This species ferments the same sugars as *C. tropicalis* but the auxanogram with ammonium sulphate is negative. It is not virulent for rabbits and produces filaments with great difficulty.

Langeron and Guerra stated that the auxanogram with asparagine is negative, but our results were always positive.

Diddens and Lodder (1939) believe that *C. pelliculosa* is the imperfect stage of *Hansenula javanica* (Groenewege) or of *Hansenula anomala* (Hansen). These yeasts ferment raffinose and assimilate nitrates, whereas *C. pelliculosa* fails to show these characteristics.

We think that *C. pelliculosa* is nearer to the genus *Torulopsis* than to the genus *Candida*.

#### 5. *Candida intermedia* (Ciferri and Ashford) Langeron and Guerra, 1938

*Culture studied.* Ashford's strain received from Langeron. As stated by Conant this species is indistinguishable from *C. tropicalis* in the tests used by Martin and Jones (1940). Nevertheless, *C. intermedia* is very different from *C. tropicalis* because it grows very poorly at 37 C and is not virulent for rabbits, and because its auxanogram with lactose is positive. We think that this species is not important from a medical standpoint.

#### 6. *Candida krusei* (Castellani) Berkhout, 1923

*Cultures studied.* *Monilia inexpectata* Mazza, Niño, and Egüez; strain received from Mazza. *Candida krusei*, Mrak's strain 423 isolated from dates. Two strains isolated by Mrak from grapes (Mrak's strains 200 and 201) and one from prunes (Mrak's strain 179). One strain isolated in Montevideo from grapes and another from red wine. Two strains isolated in Bolivia by F. Veintemillas from the Indian beer named "Chicha" (strains 772 and 774 from our collection). One strain isolated in Montevideo from sputum and another from the normal skin of a man (575). One strain isolated with *C. tropicalis* from a case of bronchomoniliasis in Montevideo. One strain received from Langeron.

The growth on solid medium is flat, dry, or with slight brilliancy during the first days. In liquid media all the strains produced a characteristically smooth or faintly corrugated surface film which extended up the sides of the tube to a distance of 5 to 8 mm above the surface of the liquid.

True mycelium was never observed. The pseudomycelium consisted of hyphal elements, usually from 5 to 50  $\mu$  long (figures 5 and 6). The distal end of each element produced blastospores which tended to become oval or elongated. If the blastospores were short, a mycotoruloides type of growth was observed, but if they became elongated, treelike mycelial structures were observed. We have never seen chlamydospores.

The auxanographic method did not produce valuable information about the utilization of sugars, and we were obliged to use Laurent's liquid medium. In this medium, with 2 per cent glucose, we observed the formation of a thick film; with maltose and galactose a thin film was observed; and no film was produced with sucrose, lactose, or raffinose. Consequently we think that *C. krusei* utilizes glucose, maltose, and galactose.

Langeron and Guerra, on page 53 of their excellent paper, stated that a strain named *Monilia inexpectata* produced four-spored asci. Talice and Mackinnon (1934) had named this strain *Mycocandida inexpectata* (Mazza, Niño, and Egüez). On page 483 Langeron and Guerra declared that they had studied a new strain of *Mycocandida inexpectata*, received from Talice and Mackinnon, which did not produce asci; this strain was considered identical with *C. krusei*. It is quite evident that Langeron and Guerra believed that they had worked with two different strains, but we have had in our laboratory only one culture of *Monilia inexpectata*, which we later named *Mycocandida inexpectata*. Consequently, Langeron and Guerra observed asci once and did not observe asci any other time in the same strain, which they considered as *C. krusei*. We have carefully investigated the production of asci in the strain named *M. inexpectata* and we have never found them, but we found asci in another strain which we have identified as *C. krusei* and which was isolated by us from normal skin. The asci were extremely scarce and showed four round spores. We think that *C. krusei* is able to produce ascospores only under rare conditions.

Some of our strains were isolated from wine and fermented liquids. Had we not had a medical mycological training we should have recorded these strains as *Mycoderma cerevisiae* Desmazières. In the genus *Mycoderma sensu* Leberle, we believe that Lodder includes species whose microscopic appearances are very similar to those of *C. krusei*. Nevertheless, these species would fail to ferment sugars; on the other hand, Guilliermond (1920) stated that some strains of *M. cerevisiae* produce small amounts of alcohol. Our strains named *C. krusei* fermented glucose very slowly, sometimes only after 10 days, and they always produced small amounts of gas, only one or two ml. In 1 per cent glucose medium we very often could not observe the production of gas. We believe it is not possible to distinguish *C. krusei* from *Mycoderma cerevisiae*. Stovall and Bulbolz (1932) also stated that *Monilia krusei* was a *Mycoderma*.

The finding of asci by Langeron and Guerra and by us in two strains is very important. These two strains could be placed in the genus *Pichia* Hansen, and very close to the species *Pichia alcoholophila* Kloecker. Stelling-Dekker (1931) said that *P. alcoholophila* does not ferment glucose, whereas Kloecker stated that it has a low power of fermentation of glucose; yet both Stelling-Dekker and

Kloecker dealt with the same strain. Stelling-Dekker did not find asci, but Kloecker described asci. This discordance may be explained by the loss of a characteristic or by the fact that the asci are produced only under very exceptional conditions.

Consequently, we consider that the imperfect stage of *Candida krusei* may be identified with a *Mycoderma* and that the perfect stage belongs to the genus *Pichia*.

#### 7. *Candida parakrusei* (Castellani) Langeron and Guerra, 1938

*Cultures studied.* *Monilia parapsilosis*, Ashford's strain. *Mycocandida parakrusei*, strain received from Langeron. *Monilia onychophila*, Pollacci and Nannizzi's strain received through Langeron. One strain received from P. Negroni (Argentina). One strain received from G. B. Schouten (Paraguay) and isolated from the vagina of a woman. One strain isolated from the normal skin and another from a case of onychia in Montevideo.

This is a well-known species. It does not produce true mycelium and chlamydospores and is not virulent for rabbits. We did not observe dissociation or spontaneous hereditary changes. The auxanogram with urea is negative when the medium is prepared with new bacto agar and positive when reclaimed agar is used.

#### 8. *Candida guilliermondi* (Castellani) Langeron and Guerra, 1938

*Cultures studied.* *Monilia guilliermondi*, Castellani's strain. One strain received from Negroni with the name *Candida chalmersi*, and one strain isolated from sputum in Montevideo.

On solid medium the colonies are smooth and lustrous, but sometimes a few radial furrows may be seen. Langeron and Guerra described a veil in liquid medium; we observed neither veil nor ring. They also stated that *C. guilliermondi* does not utilize maltose; we obtained positive results. It is rather difficult to obtain formation of filaments on slide cultures. One strain produced good filaments with verticils of small blastospores about 1.5 to 2.5  $\mu$  in diameter, but we also observed larger blastospores.

#### 9. *Candida chalmersi* (Castellani) Basgal, 1931

*Cultures studied.* *Candida chalmersi*, Mrak's strain 124 isolated from Kadota figs. *Candida chalmersi*, Mrak's strain 210 isolated from dates.

The cultures on solid medium are creamy white and become darker with age; they are soft, smooth, and lustrous, but after 2 to 4 weeks thin and irregular furrows may appear. In liquid media a smooth and dry veil is formed.

It is difficult to obtain filaments on slide cultures, but in the depth of the solid medium we could study treelike structures. Some strains produce abundant pseudoconidia which may form a sheath around the filaments. We also found several round and thick-walled cells about 6 to 7  $\mu$  in diameter. We believe these cells to be chlamydospores.

We were able to dissociate one strain (culture 813) into two variants. One

variant differs from the other in that it produces more filaments and the cultures on solid medium are rougher; we believe that this variant may be related to an "R" form.

Some authors think that *C. chalmersi* is a synonym of *C. parakrusei*, but *C. parakrusei* does not ferment sucrose nor produce a film on the surface of liquid media. Mrak, Phaff, and Vaughn (1942) stated that their strains utilized urea and fermented raffinose; a strain obtained from Langeron also utilized urea, although Langeron and Guerra stated that urea was not utilized. The strains which we have studied were received from Mrak and utilized neither urea nor raffinose. The differences between our results and those of Mrak, Phaff, and Vaughn may be due to the different quality of the products with which we have experimented.

#### 10. *Candida macedoniensis* (Castellani) Berkhout, 1923

*Culture studied.* Castellani's strain received from Negroni. Diddens and Lodder created the new designation *Saccharomyces macedoniensis* for the perfect stage (1939) of this organism.

The growth on solid medium is soft, shiny, smooth, creamy white. The edges are translucent. On liquid medium, neither veil nor ring is produced. The central portion of the cultures consists of round or oval blastospores (2 to 6 x 3 to 4  $\mu$ ). The periphery shows abundant pseudofilaments which may form microscopic, coremiumlike structures. These filaments produce few blastospores and are levogyre.

*C. macedoniensis* produces acidification and clotting of milk; it utilizes inulin but not maltose. Castellani (1937) and Diddens and Lodder (1939) say that it ferments inulin, but our results were negative.

#### 11. *Candida pseudotropicalis* (Castellani) Basgal, 1931

*Culture studied.* *Candida mortifera*, Redaelli's strain received from Langeron.

On solid medium the growth is soft, flat, smooth and shiny, and creamy white. In liquid medium neither a veil nor a ring was observed. On slide cultures the filaments produce treelike structures. Langeron and Guerra stated that galactose was not fermented, but our results were positive; this difference is not an important one. Diddens and Lodder (1939) demonstrated that *C. pseudotropicalis* should be considered as the imperfect stage of *Saccharomyces fragilis* Jörgensen.

#### 12. *Candida brumpti* (Langeron and Guerra) Langeron and Guerra, 1938

*Cultures studied.* *Blastodendron brumpti*, Langeron's strain. *Candida ravanti*, Langeron's strain.

The growth on solid medium is smooth, shiny, soft, and with an undulant edge which becomes filamentous at the upper part of the tube. In liquid media a thin film is observed. On slide cultures we were unable to obtain good filaments. In old cultures on Sabouraud's agar we usually observed good filaments; we found a true mycelium and a pseudomycelium about 1.75 to 2.5  $\mu$

in diameter. The blastospores are produced by the sprouting of the filaments; sometimes they form short chains. A branch of the mycelium may give rise at its end to 2 or 3 chains of blastospores. Some blastospores are rather elongated. Verticils of blastospores may also be occasionally observed. Some filaments produce pseudoconidia. We also found chlamydospores about 6 to 10  $\mu$  in diameter.

Langeron and Guerra observed smooth colonies of round or oval cells and corrugated colonies consisting of elongated cells. We were able to dissociate a strain and obtained smooth and rough cultures. Langeron and Guerra stated that *C. brumpti* has a weak fermenting power; our results were negative.

Some authors believe that *C. brumpti* is a synonym for *C. parakrusei*. We do not agree because *C. brumpti* does not ferment glucose; it does not grow at 37 C; it does not utilize sucrose; and it produces true mycelium and chlamydospores.

### 13. *Candida flareri* (Redaelli and Ciferri) Langeron and Guerra, 1938

*Culture studied.* One strain isolated from a case of paronychia in Montevideo, together with *Rhodotorula mucilaginosa*.

The cultures on solid medium form a thick, soft, and creamy white film. This growth is smooth during the first days, but very soon some radiating and superficial furrows appear. These furrows limit sectors which vary in depth of pigmentation. After several weeks the center of the giant colonies showed some verrucosity. Filaments may be seen occasionally around the upper parts of the cultures. In liquid medium a partial mucous veil may be observed. We could not obtain filaments on slide cultures. On Sabouraud's agar, we sometimes observed a branched pseudomycelium which recalls the "mycocandida" type of Langeron and Talice. Some filaments show terminal chains of blastospores. Our strain did not ferment any sugar. The auxanogram with sugars is identical with that observed by Langeron and Guerra. In Laurent's medium we obtained the same results only when thiamine was added to the medium. In Laurent's medium without thiamine, no differences were observed between the tubes with sugars and those without sugars. This experiment demonstrates that our strain has a thiamine requirement. The auxanogram with urea was negative, but when we added to the medium heated cells of *Saccharomyces cerevisiae* or of *Rhodotorula mucilaginosa* (from a culture on Sabouraud's agar), we obtained a positive auxanogram for urea. The addition of thiamine instead of yeasts did not produce the same results. These experiments seem to show that our strain has several deficiencies.

Our strain was isolated from onychia. The cultures of this case produced two different types of colonies: (1) numerous red colonies which were identified with *Rhodotorula mucilaginosa*; (2) a few white colonies which we have identified as *C. flareri*. *R. mucilaginosa* may produce mucoid, smooth, or rough colonies, and we have observed that even the mucoid or normal form may produce filaments similar to those we observed in *Candida flareri*.

Nadson and Philippov (1928) obtained from *Rhodotorula glutinis* races which were characterized by discoloration. We have obtained from *R. mucilaginosa* pale races.



Our experiments show that *R. mucilaginosa* and *C. flareri* utilize exactly the same sugars. The auxanograms are identical even when a minor detail, such as the weak assimilation of galactose, is taken into consideration. In Laurent's liquid medium it was necessary to add thiamine in order to obtain good growth of *R. mucilaginosa*. The utilization of urea by *R. mucilaginosa* was negative in the synthetic medium recommended by Lodder, but when dead cells of the same strain (from cultures on Sabouraud's agar) were incorporated in the medium, the auxanogram with urea became positive. Moreover, *R. mucilaginosa* may produce some long and filamentous growth in the depth of the agar.

It is evident that many similarities exist between the strain which we called *Candida flareri* and the species *Rhodotorula mucilaginosa*. The only difference which we found is that *C. flareri* is white whereas *R. mucilaginosa* is red because of a carotinoid pigment; but this characteristic of the *Rhodotorulaceae* may be lost as we have seen above. Moreover, we must not forget that the only strain of *C. flareri* which we have isolated was found together with *R. mucilaginosa*. All these facts suggest that *C. flareri* is an unpigmented variant of the "S" form of *R. mucilaginosa*.

#### 14. *Candida suaveolens* (Lindner) Langeron and Guerra, 1938

*Culture studied.* *Neogeotrichum pulmoneum*. Magalhães; the Magalhães strain received from Arêa Leão.

We have identified with this organism the strain named *Neogeotrichum pulmoneum* or *Oidium brasiliense* by Magalhães. Magalhães claims that this species produces bronchomycosis (1918, 1929, 1932). The colonies grow rapidly. The cultures on glucose agar are cream-colored; they have a membranous consistency and show deep and irregularly arranged furrows which produce a cerebriform aspect. In liquid media a membranous veil is produced. Filaments are easily obtained on slide cultures. The pseudofilaments (2 to 3  $\mu$  in diameter) are built up into short and long segments. The short segments predominate at the end of the pseudofilaments. Each segment sprouts at its distal end and produces 2 or 3 blastospores. The segments of the filaments adhere one to another and may simulate arthrospores. The branches of the filaments adhere also to the main filament and sometimes the general aspect is that of a feather that has been introduced into water (figures 7, 8, and 9).

The biochemical properties are summarized in table 1. We also observed liquefaction of gelatin. The following tests were negative: liquefaction of Loeffler's serum, production of indole and ammonia, reduction of nitrates, hydrolysis of starch, and the Voges-Proskauer test.

Langeron and Guerra described mucoid, smooth, and membranous colonies and observed several changes. The strain of Magalhães has been studied in the "R" or membranous form.

Puntoni considers that *C. suaveolens* is a *Trichosporon*. We think that this is not justified because *C. suaveolens* does not produce true arthrospores.

We have asked several laboratories for strains of *Neogeotrichum pulmoneum* and received seven cultures which we identified as *Candida tropicalis* (four

strains), *C. albicans* (one strain), *Geotrichum lactis* (one strain), and *Candida suaveolens* (one strain). We think that *Candida suaveolens* cannot be con-



FIG. 7. *CANDIDA SUAVEOLENS* (OR *NEOGEOTRICHUM PULMONEUM* OR *OIDIUM BRASILIENSE*). MAGALHÃES STRAIN. SLIDE CULTURE. MAGNIFICATION:  $\times 95$

FIGS. 8 AND 9. *CANDIDA SUAVEOLENS*. MAGALHÃES STRAIN. SLIDE CULTURES. MAGNIFICATIONS:  $\times 570$

sidered as a cause of bronchomycosis; it has been confused with other species which have a true pathogenic action, namely, *C. tropicalis* and *C. albicans*.

15. *Candida deformans* (Zach) Langeron and Guerra, 1938

We did not get any strain. Langeron and Guerra described membranous, wrinkled cultures. On liquid medium a membranous veil was observed. Filaments are easily observed and show some treelike structures with pleurogenous (not verticillate) branches which look like a true mycelium.

16. *Candida zeylanoides* (Castellani) Langeron and Guerra, 1938

We could not study any strain of this species. Langeron and Guerra state that the colonies are creamy white, soft, shiny, and smooth, but sometimes they show sectors with furrows. A filamentous halo may be observed around some colonies. On liquid media, a partial veil is formed. On slide cultures the filaments are not easily obtained. The biochemical properties are summarized in table 1.

## SUMMARY AND CONCLUSIONS

The auxanographic methods of the European authors (Stelling-Dekker, Lodder, and Langeron and Guerra) are necessary for a correct identification of the pathogenic as well as the nonpathogenic yeasts.

The genus *Candida* Berkhout is a heterogeneous group of species.

The existence of true dissociation or hereditary changes in some species of the genus *Candida* is a fact which cannot be denied. One type of variation may be related to the "R" form of bacteria ("membranous variant" of Mackinnon), and another type of variation observed in *Candida albicans* to the "lethal races" of Krassilnikov and of Nadson, observed in *Saccharomyces cerevisiae*.

The perfect stage of some species has been found: Diddens and Lodder demonstrated that *Saccharomyces fragilis* is the perfect form of *Candida pseudotropicalis*; the same authors called *Saccharomyces macedoniensis* the perfect form of *Monilia macedoniensis*.

*Candida krusei* also has a perfect stage. The imperfect form of this species cannot be distinguished from *Mycoderma cerevisiae* Desmazières, and the perfect form must be included in the genus *Pichia* Hansen.

*Candida tropicalis* is a very important species for the medical pathologists. Some strains produce abundant true filaments. These filamentous strains may produce arthrospores, even appressoria. We did not observe chlamydospores.

*Candida intermedia* is not a synonym of *C. tropicalis* and we think that it does not deserve medical interest.

*Candida pelliculosa* seems to be a *Torulopsis*.

We think that *Candida flaveri* (Redaelli and Cerri) is a white variant of *Rhodotorula mucilaginosa* (Jørgensen) Harrison.

*Candida albicans* (Robin) Berkhout, and *Candida stellatoidea* (Martin, Jones, Yao, and Lee) show some common characters, as well as characteristic and identical chlamydospores. They produce a true mycelium. Both species easily produce "membranous" or "R" variants.

We think that *Neogeotrichum pulmonicum* Magalhães (*Oidium brasiliense* of the same author) is a synonym for *Candida suarcolens*. This species has been found in a few cases. But seven strains related to *N. pulmonicum* have been

identified in our laboratory with *Candida tropicalis* (four strains), with *Candida albicans* (one strain), with *Geotrichum lactis* (one strain), and with *Candida suaveolens* (the original strain).

We agree with Conant's claim that *Syringospora* Quinquaud was the first validly published genus for these yeasts. We think there cannot be any doubt that *Syringospora* is the first validly published genus for the agent of thrush (*Candida albicans*) and for the related species, *Candida stellatoidea*.

Some species like *C. albicans*, *C. stellatoidea*, and *Candida parakrusei* are common parasites of the mucous membranes, which seem to be the normal habitat. Other species, like *Mycoderma cerevisiae* (*Candida krusei*), *Saccharomyces fragilis* (*Candida pseudotropicalis*), and *Candida chalmersi*, are saprophytes outside the human body.

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# HEAT ACTIVATION INDUCING GERMINATION IN THE SPORES OF THERMOTOLERANT AND THERMOPHILIC AEROBIC BACTERIA

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Relatively mild heating of the spores of mesophilic aerobes has been shown to hasten their subsequent germination (Evans and Curran, 1943). Although effective in bringing about a more rapid germination, with one exception, such heating had no measurable influence upon the number of spores that germinated. This tangible evidence of pregermination stimulation led us to extend our observations to a group of thermotolerant aerobes isolated from commercially canned evaporated milk that had spoiled. With nearly all the latter types, preheating was found to exert a determining influence upon the number of spores that germinated. This phenomenon, which must be regarded as true heat activation, has not been reported previously for bacterial forms, although evidence of its operation may be found in the publications of Mudge and Thorwaldsen (1930) and Christian (1931), both of which dealt with obscure milk defects in which heat affected the development of sporeforming organisms. Seeking to explain observed qualitative and quantitative changes in the flora of pasteurized milk, Mudge and Thorwaldsen formulated an interesting hypothesis which assumes for certain thermophiles a complex life cycle involving both visible and invisible spore forms. The latter, normally dormant, might be induced to germinate by physical and chemical agencies, including heat. Christian believed that heating promoted the development of a "coconut" or "carbolic" taint in commercial sterilized milk by destroying a product of vegetative activity inhibitory to the germination of the spores.

Some light is shed upon the heat activation reaction by experiments reported in this paper. The relationship between the amount of pregermination heat and the degree of activation, and certain factors exclusive of pregermination heat which affect the heat response are given especial consideration.

## METHODS AND MATERIALS

Observations were made on the following organisms: 15u, 4149, 6 (American Can Company); CON ("A," Continental Can Company); 9499 (National Canners Association); G<sub>1</sub> and H<sub>2</sub> (Whitehouse Evaporated Milk Company); LB (Bureau of Dairy Industry); *Bacillus coagulans* (Iowa State College), a thermotolerant aerobe isolated from spoilage in commercially processed evaporated milk; *Bacillus calidolactis*, 2 strains, one from Iowa State College and 1518 from the National Canners Association, both obligate thermophilic aerobes recovered from spoiled evaporated milk and corn, respectively; *Bacillus subtilis* 6051 and 6634 (American Type Culture Collection); and 3679 (American Can Company),

a putrefactive mesophilic anaerobe. Cultures 6, 15u, 4149, and LB were subsequently examined by Doctor Ruth Gordon of the staff of the American Type Culture Collection and provisionally identified as *Bacillus subtilis*.

Plain nutrient agar slopes (pH 7.0) were used as mediums in the production of the spores unless otherwise stated. The incubation temperature was 37 C, except for 1518 which usually was 55 C. When sporulation was complete (2 to 3 weeks' incubation) the growth was washed into sterile distilled water, filtered through cotton, and centrifuged. The water was decanted and the washing process repeated 3 times. The washed spores were then filtered through the cotton plunger tube as described by Morrison and Rettger (1930), which effectively removed most of the clumps. The concentrated stock suspensions thus prepared, practically 100 per cent spores (except 1518), were plated to determine purity and count, and were then held at 6 C until used.

The nutrient agar (pH 7.0) used as a plating medium contained, per liter: peptone (Difco) 5 g, beef extract (Difco) 3 g, sodium chloride 5 g, glucose 3 g, and agar 13 g.

The general plan of the experiments was as follows: small aliquot portions of the stock suspensions diluted in distilled water were seeded into 8-ml quantities of the test medium contained in pyrex tubes. After thorough mixing, selected tubes were set aside to serve as controls, and the remainder were heated in a 95 C bath, usually for 10 minutes. The heating was accomplished in a stirred glycerol bath equipped with a thermoregulator which maintained the desired temperature at  $\pm 0.5$  C. The heated and unheated control samples were then plated on glucose agar of the composition noted above. Routinely, plates were counted after 48 hours of incubation, and at regular short intervals counted plates were returned to the incubator and re-examined after 4 additional days of incubation. When low subcultivation temperatures were used, the incubation periods were necessarily extended, as indicated in the tables or text.

#### EXPERIMENTAL

*Activation of spores in relation to the amount of heat applied.* The proportion of spores that respond to pregermination heating is dependent upon the amount of heat applied. This is revealed in the following experiment in which a suspension of spores was subjected to different periods of heating at constant temperatures. When the spores were uniformly dispersed in sterile skim milk plated both immediately and after varying periods of heating at 95 C, the effect of heating was to increase materially the number of colonies which developed. The results obtained with 3 members of the spoilage group are shown in figure 1. The incubation temperature of the plates was 37 C. The colony count increased with the time of heating rather rapidly during the first few minutes, then more slowly until a maximum was reached, usually between 15 and 30 minutes; longer heating reduced the number of colonies. It is clear that, under these conditions of production, treatment, and subcultivation, germination of the spores is a function of the amount of heat to which they have been exposed. Without some preheating only a small proportion of the potentially viable spores were able to

germinate; prolonging the incubation time, varying the nutritional character of the plating medium, and other measures, except temperature elevation, had no appreciable influence upon the number of colonies formed. The rising slope of the curve shows that the heat activation threshold differs with the individual spores in a culture.

*Activation of spores as influenced by the nature of the heating medium and by the temperature of subcultivation.* The germination response following the heating of spores in a 95 C bath for 10 minutes in each of 9 different mediums is shown in table 1. The results obtained at different temperatures of subcultivation reveal the importance of this factor in determining the magnitude of the heat response. The figures within the parentheses indicate the ratio of the count to that of the unheated control. With subcultivation at 37 C, preheating increased the count of the thermotolerant species (number of spores which

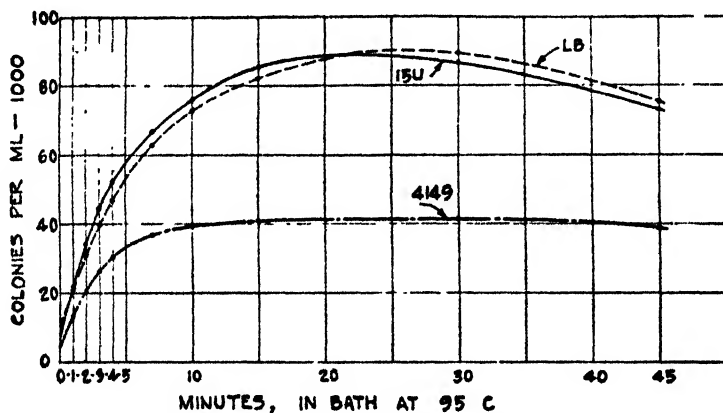


FIG. 1. THE GERMINATION OF SPORES BEFORE AND AFTER VARYING PERIODS OF MILD HEATING IN SKIM MILK

germinated) in all the heating mediums; but activation was especially pronounced in the presence of glucose, lactose, peptone, and milk. Glucose is fermented by these organisms; lactose, not vigorously attacked, promotes heat activation to about the same degree as glucose. Salt depressed heat activation whereas beef extract enhanced it moderately, as did agar alone. The results obtained in the complete agar and broth mediums reflect the balancing influence of components which both enhance and depress heat activation. Subcultivation at 48 C increased the count over that at 37 C of both the unheated spores and those heated in water, beef extract, and salt solution, but it had little or no effect in the presence of glucose, lactose, or milk. Less extensive data for the obligate thermophile (1518) show similar relationships. High temperatures of subcultivation serve to equalize the count at high levels by inducing a secondary activation of the spores in the subculture medium, a reaction which does not occur at 37 C or below. The test substances have no measurable effect upon the count in the absence of preheating or high temperature incubation. In table 2 is shown the heat activation reaction in skim milk with sub-



TABLE 1  
*The germination of the spores of thermotolerant and thermophilic aerobes after sublethal heating, as influenced by the nature of the heating medium and the temperature of subcultivation*

OR- GAN- ISM	TEMP OF SUBCUL- TIVATION	NOT HEATED	HEATED AT 95 C FOR 10 MINUTES IN									
			Distilled water	Beef extract 0.5%	Salt (NaCl) 0.5%	Peptone 0.5%	Glucose 0.5%	Lactose 0.5%	Agar 1.5%	Beef-extract peptone glucose salt agar*	Beef-extract peptone glucose salt broth*	Skim milk
	C	per ml 000	per ml 000	per ml 000	per ml 000	per ml 000	per ml 000	per ml 000	per ml 000	per ml 000	per ml 000	per ml 000
15u	37	24	83 (3X)	110 (4X)	62 (2X)	241 (10X)	195 (8X)	245 (10X)	158 (6X)	178 (7X)	95 (3X)	194 (8X)
15u	48	110	166 (1X)	215 (1X)	131 (1X)	231 (2X)	215 (1X)	233 (2X)	236 (2X)	247 (2X)	237 (2X)	220 (2X)
4149	37	82	125 (1X)	168 (2X)	145 (1X)	374 (4X)	565 (6X)	461 (5X)	250 (3X)	257 (3X)	205 (2X)	377 (4X)
4149	48	296	405 (1X)	517 (1X)	435 (1X)	500 (1X)	573 (1X)	487 (1X)	500 (1X)	520 (1X)	496 (1X)	463 (1X)
LB	37	106	240 (2X)	530 (5X)	120 (1X)	1,320 (12X)	1,790 (16X)	1,540 (14X)	510 (4X)	590 (5X)	400 (3X)	980 (9X)
LB	48	1,060	1,550 (1X)	1,470 (1X)	1,140 (1X)	1,780 (1X)	2,150 (2X)	1,610 (1X)	1,300 (1X)	1,640 (1X)	1,440 (1X)	1,160 (1X)
1518	45	9.3	51 (5X)									75.8 (8X)
1518	55	22.8	56 (2X)									77.0 (3X)

\* Component concentrations as used individually.

Nos. 15u, 4149, and LB—spores produced at 37 C.

No. 1518—spores produced at 45 C.

cultivation of the spores at temperatures down to 14 C. When the subcultivation temperatures were below 30 C, a preliminary count was regarded as final if incubation for an additional week at the same temperature resulted in no new colonies. These data reveal the important fact that preheating insures a high percentage of germination at very low temperatures. In the heated samples colonies appeared earlier and developed more rapidly than those from unheated

TABLE 2

*The germination of the spores of thermotolerant aerobes after sublethal heating as influenced by the temperature of subcultivation*

ORGANISM	TEMPERATURE OF SUBCULTIVATION	TREATMENT OF SPORES	
		Not heated	Heated at 95 C for 10 minutes
	C	per ml	per ml
15u	37	4,700	87,000
	30	3,500	85,000
	25	2,200	88,000
	20	2,400	88,000
	14	1,080	19,000
4149	37	13,000	39,000
	30	14,600	36,000
	25	15,100	33,000
	20	14,900	31,000
	14	10,500	29,000
LB	37	3,100	47,000
	30	1,700	45,000
	25	1,600	37,000
	20	1,500	37,000
	14	850	31,000

Heating medium, sterile skim milk.

37 C plates counted after 3 and 7 days' incubation.

30 C plates counted after 3 and 7 days' incubation.

25 C plates counted after 2 and 3 weeks' incubation.

20 C plates counted after 3 and 4 weeks' incubation.

14 C plates counted after 4 and 5 weeks' incubation.

samples; this lowering of the temperature of germination for the majority of spores is associated with mild heating involving no measurable lethal action.

Of considerable practical significance would be experimental evidence to prove that the relatively few spores which survive drastic heating react similarly to different subcultivation temperatures. This evidence is provided in table 3, which shows that the overwhelming majority of spores which survive severe heating germinate at lower temperatures than do unheated spores. Graphic representation of these data in terms of the percentage of germination emphasizes this fact (figure 2). It should be noted, however, that at the lowest temperature of subcultivation (14 C) none of the spores that survived autoclaving were able

to develop whereas a small proportion of the unheated spores could. Although a selective elimination of spores which germinate at the higher temperatures is not excluded, the reaction appears more likely to be a further manifestation of heat activation. A recent paper of Williams and Reed (1942) has a bearing on this subject. These authors recorded greater thermal death times for spores of *Clostridium botulinum* types A and B and of an unidentified putrefactive anaerobe (3679) when the recovery cultures were incubated at 24 and 27 C than when incubated at 31 or 37 C, although the optimal growth temperature of these organisms is in the 35 to 37 C range. Working with the spores of the same putrefactive anaerobe (3679), we obtained some evidence for heat activation in this species, but the results were deemed inconclusive. (The special Brewer petri

TABLE 3

*The germination of the spores of thermotolerant aerobes after severe heating in skim milk as influenced by the temperature of subcultivation*

ORGANISM	TEMPERATURE OF SUBCULTIVATION	TREATMENT OF SPORES	
		Not heated	Heated at 120 C; autoclaved for 10 minutes
15u	C	per ml	per ml
	48	980,000	30
	37	129,000	1,900
	30	83,000	2,650
	25	81,000	2,050
	20	86,000	450
	14	6,100	0
LB			6 minutes
	48	3,470,000	10
	37	254,000	2,850
	30	218,000	4,270
	25	160,000	3,000
	20	133,000	900
	14	71,000	0

dish cover and B-B-L anaerobic agar were used.) The net result of preheating, provided it is adequate, is to lower the temperature at which germination of the majority of spores can occur. It would appear that these spore populations are divisible into several classes or groups depending on their minimal temperatures for germination—the largest, with a relatively high temperature minimum, is susceptible to heat activation, which enables the spores to germinate at sub-minimal temperatures.

Table 4 shows the effect of different concentrations of glucose and of salt upon the heat activation of spores; for each of the 3 cultures, glucose in 0.1 per cent concentration was most effective in promoting heat activation. The depressing action of salt upon heat activation is evident in the higher concentrations.

*The temperature at which spores are formed in relation to heat activation.* In this experiment spores of 15u, 4149, and LB, produced at 30, 37, 45, and 52 C

were heated in milk at 95 C for 10 minutes, after which they were subcultivated on plates at 37 and 48 C. Spores of the obligate thermophile (1518) produced at 45, 50, 55, and 65 C were similarly heated, but subcultivated at 45, 55, and 61 C. The data are shown in table 5. In three of the cultures (15u and LB, and 1518), activation was greatest at 45 and 55 C, respectively—temperatures which approximate the optimal for growth. With 4149, spores produced at suboptimal temperatures were most responsive to heat. As in the experiment which in-

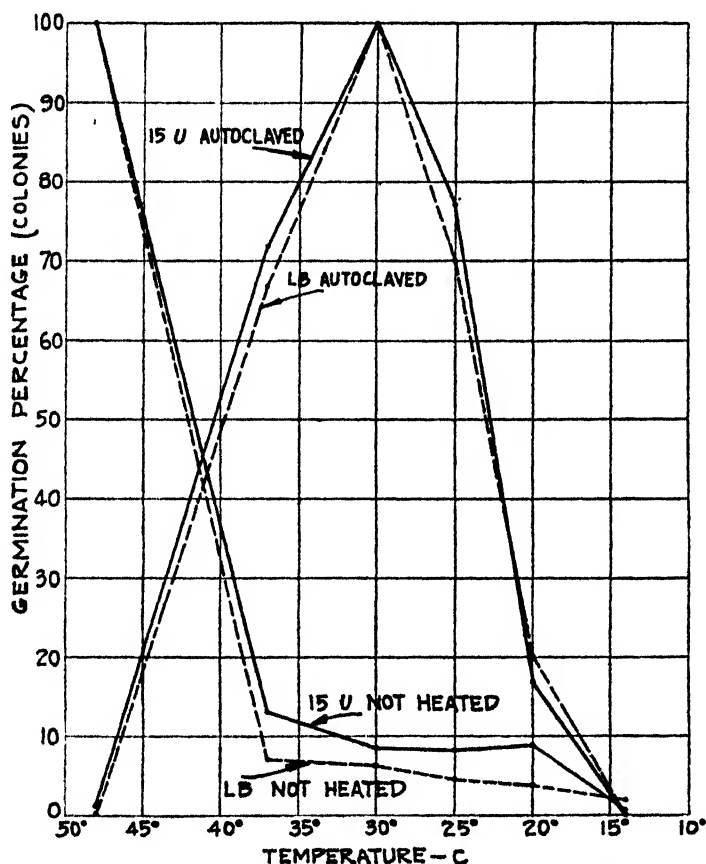


FIG. 2. THE PERCENTAGE GERMINATION OF SPORES AT DIFFERENT TEMPERATURES BEFORE AND AFTER AUTOCLAVING (120 C FOR 10 MINUTES) IN SKIM MILK

involved the different heating mediums (table 1), the higher temperatures of subcultivation effect a smoothing out of count differences, both between heated and unheated samples and among the heated samples incubated at different temperatures. The probable explanation for this is that heat at 48 C, but not at 37 C (temperature of subcultivation), maintained for 48 hours (incubation time), is itself sufficient to activate many of the spores—a reaction which, as has been shown, would be promoted by the peptone and glucose constituents of the medium. Similarly some activation of spores must also occur when they are formed at high temperatures. At 52 C spore production in 15u, 4149, and LB was much

reduced over that of 37 and 45 C. Although lower in their susceptibility to heat activation than those produced at lower temperatures, they and the spores (1518) produced at 65 C do nevertheless respond in some degree to preheating.

TABLE 4

*The germination of the spores of thermotolerant aerobes after sublethal heating as influenced by the concentration of glucose and of salt in the heating medium*

ORGAN-ISM	TREATMENT OF SPORES									
	Not heated	Heated at 95 C for 10 minutes in								
		Glucose 0.01%	Glucose 0.05%	Glucose 0.1%	Glucose 0.5%	Glucose 1.0%	NaCl 0.05%	NaCl 0.1%	NaCl 0.5%	NaCl 1.0%
	<i>per ml</i> 000	<i>per ml</i> 000	<i>per ml</i> 000	<i>per ml</i> 000	<i>per ml</i> 000	<i>per ml</i> 000	<i>per ml</i> 000	<i>per ml</i> 000	<i>per ml</i> 000	<i>per ml</i> 000
15u	68	850	2,050	2,110	1,090	910	243	155	121	111
4149	67	330	430	560	520	490	109	103	89	79
LB	116	840	1,590	1,760	1,580	1,470	175	171	103	75

Subcultivation temperature, 37 C.

TABLE 5

*The germination of the spores of the thermotolerant and thermophilic aerobes after sublethal heating\* as influenced by the temperature at which the spores were formed*

ORGAN-ISM	TEMPER-ATURE OF SUB-CULTIVA-TION	TEMPERATURE AT WHICH SPORES WERE PRODUCED							
		30 C		37 C		45 C		53 C	
		No heat	Heated*	No heat	Heated*	No heat	Heated*	No heat	Heated*
	C	<i>per ml</i> 000	<i>per ml</i> 000	<i>per ml</i> 000	<i>per ml</i> 000	<i>per ml</i> 000	<i>per ml</i> 000	<i>per ml</i> 000	<i>per ml</i> 000
4149	37	215	1,390 (6X)	47	211 (4X)	21	81 (3X)	0.21	0.43 (2X)
4149	48	521	1,940 (3X)	170	330 (1X)	87	97 (1X)	0.34	0.52 (1X)
15u	37	21	314 (14X)	32	400 (12X)	24	963 (40X)	0.09	0.62 (6X)
15u	48	44	435 (9X)	52	457 (8X)	54	964 (17X)	0.15	0.66 (4X)
LB	37	20	250 (12X)	39	410 (10X)	22	360 (16X)	1.88	4.76 (2X)
LB	48	94	330 (3X)	118	430 (3X)	50	310 (6X)	2.27	4.67 (2X)
		45 C		50 C		55 C		65 C	
1518	45	5.4	46 (8X)	4.2	56 (13X)	4.9	71 (14X)	5.20	17.4 (3X)
1518	55	18.3	55 (3X)	19.8	56 (2X)	17.9	67 (3X)	18.00	22.7 (1X)
1518	61	24.4	51 (2X)	27.9	66 (2X)	24.2	74 (3X)	26.40	33.0 (1X)

\* 95 C for 10 minutes in skim milk.

*Pasteurization in relation to heat activation.* Spores of 15u, 4149, and LB were seeded into sterile skim milk, heated at 62 C for 30 minutes, then subcultivated in the usual way. With subcultivation at 37 C (table 6) the count was increased at least 50 per cent with the expected lower response accompanying the higher temperature of subcultivation.

*Persistence of heat activation.* Tests with these cultures have shown that spores activated by heating remain in the activated condition for a period of months. This suggests that the heat-induced change is irreversible, which would distinguish it from a somewhat similar reaction in the ascospores of certain fungi

TABLE 6  
*Pasteurization in relation to heat activation in spores\**

ORGANISM	TEMPERATURE OF SUBCULTIVATION	TREATMENT OF SPORES	
		Not heated	Heated at 62 C for 30 minutes†
	C	per ml	per ml
15u	37	11,200	22,000
15u	48	26,000	34,000
4149	37	6,800	20,000
4149	48	25,000	32,000
LB	37	10,100	21,000
LB	48	30,000	41,000

\* Spores produced at 37 C.

† Heating medium, skim milk.

TABLE 7  
*The germination\* of the spores† of two strains of Bacillus subtilis after sublethal heating in skim milk, as influenced by the temperature of subcultivation*

ORGANISM	TEMPERATURE OF SUBCULTIVATION	TREATMENT OF SPORES	
		Not heated	Heated at 85 C for 10 minutes
	C	per ml	per ml
6051	37	38,000	39,000
	20	45,000	48,000
	14	52,000	57,000
6634	37	65,000	66,000
	20	55,000	67,000
	14	1,500	15,000

\* 37 C plates counted after 3 and 7 days' incubation.

20 C plates counted after 1 and 2 weeks' incubation.

14 C plates counted after 3 and 4 weeks' incubation.

† Produced at 37 C.

described by Shear and Dodge (1927) and by Goddard (1935). The latter found that spores became deactivated by conditions which prevented germination, necessitating a second heating, following which they germinated normally.

Sublethal heating was previously shown to accelerate the germination of certain mesophiles (Evans and Curran, 1943). With one exception, heat activation was not observed. Among the organisms studied were 2 strains of *B. subtilis*,

6634 and 6051, which grew readily on agar slopes at 55 C. Because of their tolerance to heat, the heat response of the spores of these cultures was retested, new crops being employed with subcultivation at 37 C, as formerly, and also at 20 and 14 C. The results are shown in table 7. Pregermination heating of the spores of 6051 had little influence upon the plate count at any of the three temperatures of incubation. Subcultivation at 37 C after preheating had no appreciable influence upon the number of spores of 6634 which germinated, confirming our previous observation. When, however, lower temperatures of subcultivation were used, the activating effect of heating became evident. It is noteworthy that at 14 C with the benefit of preheating, only 22 per cent of the viable spores were able to germinate; without preheating about 2.2 per cent were able similarly to develop. Thus heat activation may be operative in a culture and yet escape detection if the conditions of subcultivation are not sufficiently wide.

#### DISCUSSION

That heat activation is a factor contributing to the spoilage of canned foods of certain types can hardly be doubted. Of 12 spore forms recovered from 10 different outbreaks of spoilage in commercially processed evaporated milk, at least 10 were subject to some degree of heat activation. These include *B. coagulans* and *B. calidolactis*, well-known milk-spoilage types. With broader conditions of observation, it is not unlikely that all could be shown to respond. Flat sour types (*Bacillus stearothermophilus*) are heat-activatable.

Heat activation may be regarded as an adaptation by which a spore population is able to attain a high-percentage germination at suboptimal temperatures—temperatures which, in the absence of heating, permit the germination of only a small proportion of the total viable spores; with progressive lowering of the temperature of subcultivation a temperature range is ultimately reached at which germination of part of the heated and no germination of the unheated spores occur. Heat activation seems to be a property primarily of thermotolerant and thermophilic species that possess unusually high thermal resistance; spores of such organisms, because of the heat treatment that they have survived, are preconditioned to development at low temperatures, which greatly increases their spoilage potential. Where thermotolerant species are concerned, heat activation operates to bring the temperature of germination of the majority of the spores within the usual storage and distribution temperature range of canned foods.

Heat activation has a bearing upon certain commercial or laboratory practices, of which two may be mentioned. It is a favored and long-established practice among bacteriologists to apply sublethal heating as a means of eliminating vegetative cells from test spore suspensions prior to their use; the attendant change in the germinative capacity of the spores has not previously been suspected, and, whenever such heating has been employed prior to plating, the operator unwittingly increased the accuracy of the count. In the manufacture of evaporated milk, it is a routine practice to forewarm the raw milk at 95 C for 10 minutes prior to its condensation. This is necessary for the attainment of a

satisfactory body in the finished product. As a result of such heating, the majority of the spores of thermotolerant species become heat labile during the short period the milk is undergoing condensation (unpublished observation), and this large proportion, in consequence, is easily killed by the subsequent processing treatment. This provides an example in which an established commercial practice quite fortuitously conduces to a desired result.

Recognition of the phenomenon of heat activation and some understanding of the factors which affect it are of manifest importance to the accurate enumeration of viable sporing thermotolerant or thermophilic aerobes. This may also be true for some anaerobes but our observations on the latter are not sufficient to warrant a definite conclusion.

#### SUMMARY

Sublethal heating of the spores of many thermotolerant and thermophilic aerobes has a determining influence upon the number of spores which will germinate subsequently.

A study of 12 thermotolerant cultures isolated from 10 different outbreaks of spoilage in commercially processed evaporated milk revealed that nearly all would respond to pregermination heating. In the absence of such treatment a large proportion of the potentially viable spores did not germinate. Flat sour types (*Bacillus stearothermophilus*) were found to be heat-activatable.

The proportion of spores which responded to preheating was found to be dependent upon the amount of heat, the composition of the medium in which they were heated, the temperature at which the spores were formed, and the temperature at which they were subcultured.

The heating mediums arranged in the order of their effectiveness upon heat activation were: glucose or lactose (0.5 per cent) > peptone (0.5 per cent) > skim milk > glucose nutrient agar > beef extract (0.3 per cent) > glucose nutrient broth > distilled water > NaCl (0.5 per cent).

The concentration of glucose most favorable to heat activation was approximately 0.1 per cent. NaCl usually depressed heat activation in concentrations of 0.5 per cent and higher.

At 95 C in skim milk the heat-activating process was nearly complete in 10 minutes, entirely so in 30 minutes. Long-hold pasteurization effects material activation of spores.

The response to preheating is greater when the temperature of subcultivation is suboptimal than when it is at the relatively high optimal temperatures.

Preheating of heat-activatable spores serves to lower the minimal temperature at which germination can occur; this applies when the heat treatment is mild and nonlethal and also when it is so drastic that relatively few spores survive. The economic implications of this are discussed.

Recognition of the phenomenon of heat activation and some understanding of the factors which affect it are essential to the accurate enumeration of viable, sporing, thermotolerant and thermophilic aerobes. The relationship of heat activation to certain commercial or laboratory practices is indicated.



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# THE OXIDATION OF GLYCEROL BY STREPTOCOCCUS FAECALIS

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It has been reported that certain of the lactic acid bacteria are capable of utilizing glycerol only under aerobic conditions (Gunsalus and Sherman, 1943). Inasmuch as these organisms are devoid of cytochrome (Frei *et al.*, 1934; Farrell, 1935) and are reported to produce hydrogen peroxide during respiration, the presence of a functional respiration has been questioned. It therefore became of some importance to determine the nature of the reactions involved in the utilization of glycerol by these forms.

## METHODS

The culture used, strain 24 from the departmental culture collection, had previously been identified as *Streptococcus faecalis*, Lancefield serological group D. It was shown to utilize glycerol only under aerobic conditions (Gunsalus and Sherman, 1943) and to derive energy from this process. (Certain other strains are capable of using glycerol under both aerobic and anaerobic conditions.)

Active cell suspensions can be prepared under the conditions suggested for the group B streptococci (Wood and Gunsalus, 1942). Suspensions so prepared (usually from a 1 per cent tryptone, 1 per cent yeast extract, 0.5 per cent  $K_2HPO_4$ , and 0.1 per cent glucose medium, the cells being washed with phosphate or saline) could be employed immediately in the respiration experiments or could be stored for 2 or 3 days at refrigerator temperatures. In storage the oxidative powers were gradually lost, but the loss in activity was not comparable for all substrates tested. The glucose concentration in the growth medium could not be increased (to 0.3 per cent, for example) without marked decrease in the oxidative power of the cells, especially on such substrates as glycerol and other alcohols. There was no evidence of adaptation to glycerol. Active cell preparations could be obtained by drying washed cells *in vacuo* over anhydrous calcium sulfate (drierite). The conventional Warburg techniques were used throughout.

## EXPERIMENTAL

Data on the oxygen uptake of a cell suspension refrigerated for several hours are given in figure 1. The respiration without substrate was negligible ( $Q_{O_2}(N) = 10$ ); lactate and pyruvate were not oxidized at an appreciable rate, whereas glucose was oxidized slowly ( $Q_{O_2}(N) = 74$ ). With glycerol, however, oxygen was consumed at a rapid rate ( $Q_{O_2}(N) = 420$ ) until 1 mole was used per mole of glycerol. Thus these streptococci possess a powerful respiratory system acting upon glycerol. A  $Q_{O_2}(N)$  of 600 to 700 is not unusual.

<sup>1</sup> The authors are indebted to the members of the Department of Agricultural Bacteriology, University of Wisconsin, where preliminary work on this problem was done.

*Hydrogen Peroxide Formation*

The data of figure 1 show that 1 oxygen is utilized per glycerol. The products of the reaction were therefore studied. When higher levels of glycerol were added (figure 2), only a partial oxidation occurred. For example, when 2.5, 5, and 10 micromoles of glycerol were supplied, the oxygen used per mole of glycerol was, respectively, 0.97, 1.01, and 0.67 moles. The low value at 10 micromoles could be due to the accumulation of toxic amounts of hydrogen peroxide which then would form one of the products of the reaction.

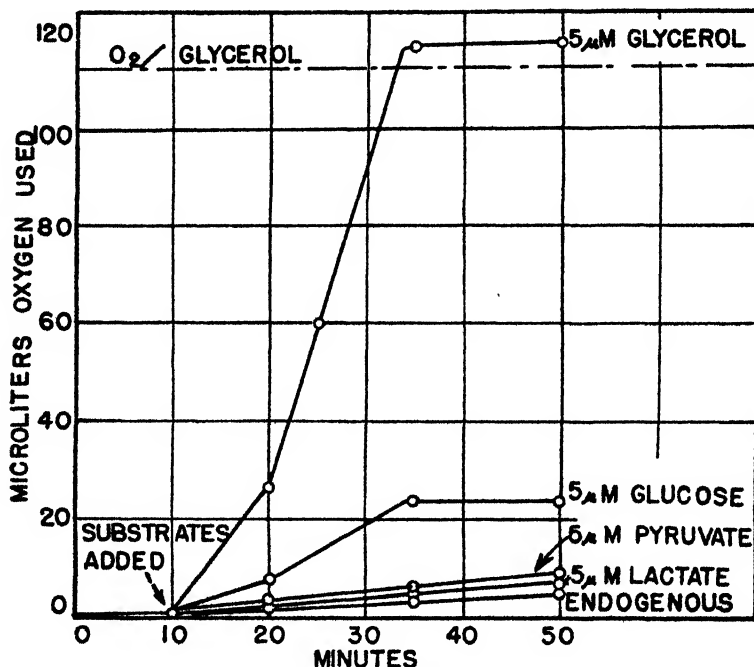


FIG. 1. OXIDATIONS BY *STREPTOCOCCUS FAECALIS*, STRAIN 24  
 0.650 mg bacterial nitrogen per cup.  
 M/30 phosphate buffer pH 7.0.  
 Total volume 3.0 ml + 0.2 ml 20% KOH in center well.  
 Temperature 37 C.

The occurrence of hydrogen peroxide was tested for in three ways.

First, by addition of catalase which would decompose the peroxide formed and thus should permit the complete oxidation of 10 or more micromoles of glycerol with an oxygen uptake of  $\frac{1}{2}$  mole of oxygen per mole of glycerol. In the presence of crystalline catalase, 1 part per 100,000 (Sumner and Dounce, 1939),<sup>2</sup> 10 micromoles of glycerol were oxidized with an oxygen uptake of 138  $\mu$ l (0.61 O<sub>2</sub>/glycerol), whereas 20 micromoles took up 223  $\mu$ l (0.50 O<sub>2</sub>/glycerol) (curves A and C, figure 3). The addition of catalase alone had no effect upon the endogenous respiration.

<sup>2</sup> We are indebted to Professor J. B. Sumner for the crystalline catalase employed.

Second, by increasing the volume of fluid in the Warburg flask to 4 ml instead of the usual 3. Thus one-third more oxygen should be taken up before the concentration of hydrogen peroxide reached an inhibitory level. On addition of 10 micromoles of glycerol to a flask with 3 ml of fluid, 138  $\mu$ l of  $O_2$  were used (46 per ml), whereas in a flask with 4 ml of fluid, 183  $\mu$ l of  $O_2$  were used (46 per ml). This would indicate that the concentration of hydrogen peroxide which inhibits the oxidation of glycerol is close to 2 micromoles per ml. The concentration of peroxide necessary to inhibit glycerol oxidation varies slightly from one suspension to the next.

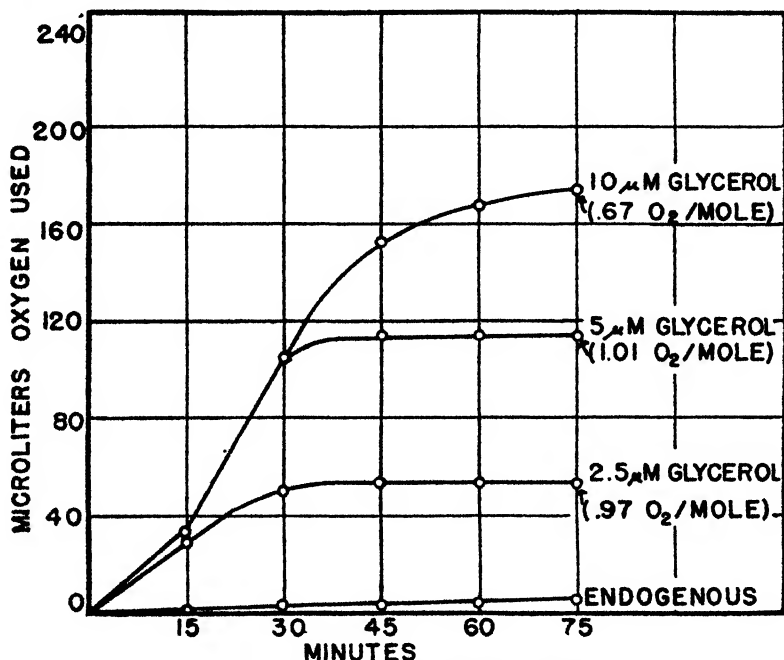


FIG. 2. GLYCEROL OXIDATION BY STREPTOCOCCUS FAECALIS, STRAIN 24  
0.45 mg bacterial nitrogen per cup.  
Other conditions as in figure 1.

Third, by the addition of pyruvate which is chemically oxidized by hydrogen peroxide to acetic acid and carbon dioxide (Sevag, 1932). This removal of peroxide would allow the oxidation of higher levels of glycerol. In the presence of 5 micromoles of pyruvate, 10 micromoles of glycerol were oxidized with the utilization of 1.02 moles of oxygen per glycerol (curve B, figure 3). This quantity of pyruvate was not sufficient to remove all of the peroxide formed, but did reduce its concentration below the toxic level. That pyruvate did not enter the reaction except to remove the peroxide is illustrated by the data in curves D and E, figure 3, which show that virtually identical amounts of oxygen were taken up by 5 micromoles of glycerol in the presence or absence of pyruvate.

Thus, one of the products of glycerol oxidation is hydrogen peroxide, which accumulates and inhibits the oxidation when its concentration reaches approximately 0.002 molar.

*Acid Production*

The other product or products of oxidation were somewhat more difficult to determine. When glycerol was oxidized in Ringer's bicarbonate buffer with an atmosphere of 5 per cent  $\text{CO}_2$  in air, one mole of acid (or acid plus carbon dioxide) was obtained per mole of glycerol. Only traces of carbon dioxide as such resulted from the oxidation. The lactic acid found, upon analysis of the flask contents by the method of Barker and Summerson (1941), accounted for only part of the acid formed. Later it became apparent that lactic acid was indeed the other product of the reaction, although in some suspensions the lactate

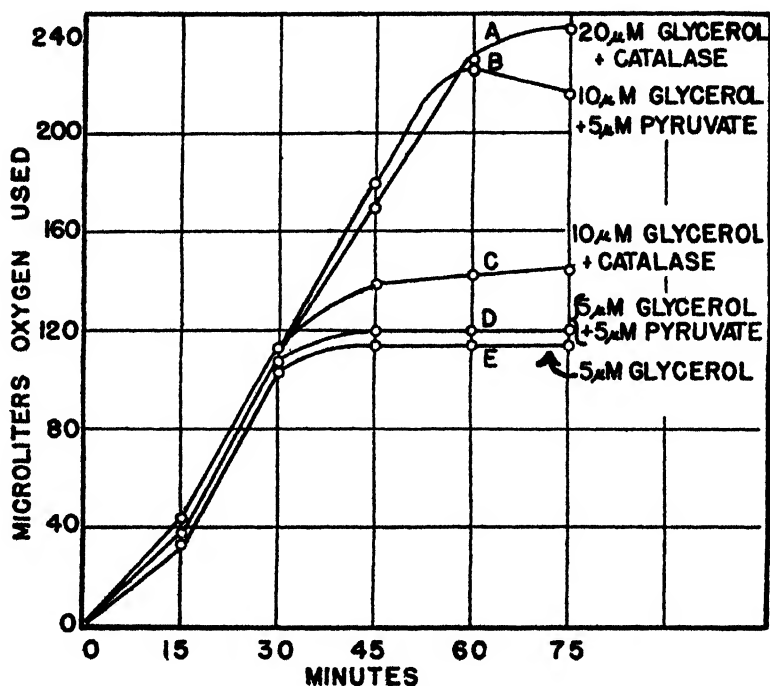


FIG. 3. GLYCEROL OXIDATION WITH REMOVAL OF HYDROGEN PEROXIDE BY PYRUVATE OR CATALASE

was further utilized (or its formation prevented) by reactions not concerned directly with glycerol oxidation. Recourse was then had to cell preparations in which some of these side reactions could be eliminated. Data upon the oxidation of glycerol with a dried cell preparation are given in figure 4. This preparation oxidized glycerol to slightly beyond the theoretical uptake of one  $\text{O}_2$  per glycerol, but was also capable of oxidizing lactate slightly. The amount of lactic acid found upon analysis, plus the amount of lactate oxidized in an equivalent time, entirely accounts for the glycerol added. With this preparation the rate of glycerol oxidation was greatly decreased in comparison with the living cell (i.e.,  $Q_{\text{O}_2}$  10.3 for the preparation as compared to values of 50 to 70 for living cells). The rate of oxidation was reduced still further by the addition of catalase

—to  $Q_{O_2}$  6.9. The concentration of hydrogen peroxide which limited the oxidation with this preparation was  $43 \mu\text{l}$  per ml as compared to  $46 \mu\text{l}$  per ml with the living cells. The over-all reaction involved in glycerol oxidation may thus be written as:



### Mechanism

It is apparent that equation (1) represents the final balance of a series of reactions leading from glycerol to lactic acid. In order to obtain information regarding the pathway of this series of reactions, the following experiments were performed. Several Warburg flasks which contained cells suspended in Ringer's buffer were prepared; ten micromoles of glycerol plus 10 micromoles pyruvate

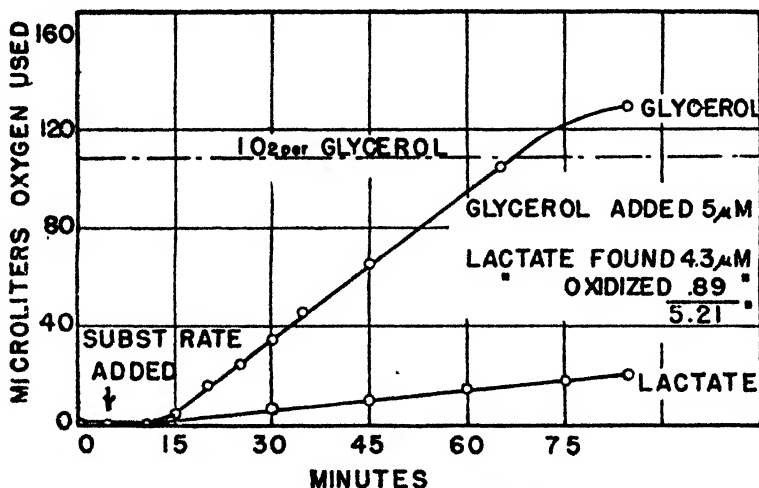


FIG. 4. GLYCEROL AND LACTATE OXIDATION BY VACUUM-DRIED CELL PREPARATIONS OF *STREPTOCOCCUS FAECALIS*, STRAIN 24

10 mg dried cell preparations per cup.  
Total volume 3.0 ml.

(to remove the peroxide formed) were added at zero time, and the course of oxygen uptake was followed. At intervals flasks were removed from the bath and 0.2 ml of 100 per cent trichloroacetic acid were added to the contents to stop the oxidation and extract the soluble phosphorous compounds from the cells. The cells were subject to extraction for 24 hours at room temperature and then removed by centrifugation, the supernatant being analyzed for inorganic phosphate by the method of Fiske and Subbarow (1925). The data in figure 5 show that the level of inorganic phosphate falls during the oxidation of glycerol, thus indicating that phosphate is esterified. After the oxidation is complete (1 mole  $\text{O}_2$  per mole glycerol) the level of inorganic phosphate rises again to approximately the starting value. Similar results are obtained if catalase is used instead of pyruvate to remove the peroxide, or if smaller quantities of glycerol are oxidized and the peroxide allowed to accumulate. No phosphate

is taken up by cells respiring endogenously. The cell preparations used in figure 4 also show a phosphate uptake. In this case  $0.13 \mu$  mole of phosphate was esterified during glycerol oxidation, whereas  $0.22 \mu$  mole was released during the same interval in the presence of lactate. Therefore, the intermediate reactions involve phosphorylation.

In the figures so far provided it will be noted that a lag of approximately seven minutes occurs from the time glycerol is added until the oxidation begins at a measurable, and maximal, rate. This suggests that the glycerol is converted into some other substance before oxidation. From the phosphate uptake one

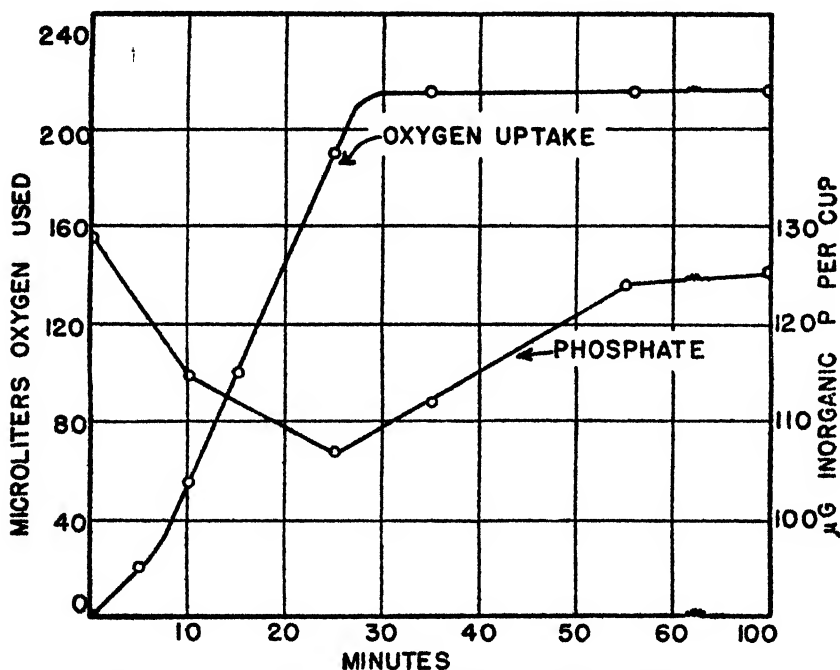


FIG. 5. PHOSPHOROUS UPTAKE DURING GLYCEROL OXIDATION

0.9 mg bacterial nitrogen.  
 $10 \mu$  moles glycerol ( $224 \mu$ l).  
 $10 \mu$  moles pyruvate, to remove  $H_2O_2$ .  
 Ringer's solution pH 7.5.

would suspect the conversion of glycerol into glycerol phosphates before oxidation, especially since glycerol-phosphate-oxidizing enzymes are known (Green, 1936; Euler *et al.*, 1937). We therefore tested purified commercial glycerol phosphate, consisting of 48 per cent  $\beta$ -, and 52 per cent  $\alpha$ -glycerol-phosphate. This mixture, however, was not oxidized at an appreciable rate by the living cell. As phosphate esters generally do not enter living cells, the ability of the dried cell preparation to oxidize the glycerol phosphates was also tested. The results were again negative. This fact seemed to exclude the glycerol phosphates as intermediates in the reaction. However, upon further study, two remaining possibilities became apparent: first, that the drying process had not destroyed

the permeability properties of the cell membrane; and, second, that the presence of the glycerol phosphates, other than the form oxidized (presumably the *l*-(-)- $\alpha$ ; see Baer and Fisher, 1939), inhibited the oxidation of the active form.

Therefore, to avoid permeability problems, studies were made with the cell preparations. A number of treatments applied to dried cells were not suitable inasmuch as they either had no effect upon the preparation, or inactivated it entirely. For example, treatment with acetone, or washing with *m*/15 phosphate at pH 5, 7, or 8 did not alter the activity. Autolysis of the preparation at 37 C for 24 hours (after which time very few cells remained) permitted the oxidation of the glycerol phosphates at a measurable rate ( $Q_{O_2} = 1.28$ ), but the preparation also oxidized glycerol ( $Q_{O_2} = 1.43$ ). Finally, preparations capable of oxidizing glycerol phosphate were obtained by grinding dried cells *in vacuo* in a micro ball mill.

TABLE 1

*Effect of varied treatments on the ability of vacuum-dried cells of Streptococcus faecalis (no. 24) to oxidize glycerol and glycerol phosphates*

TREATMENT	$Q_{O_2}$ GLYCEROL	$Q_{O_2}$ GLYCEROL PHOSPHATES	REMARKS
Living cells . . . . .	42.0	0.1	
Same cells dried <i>in vacuo</i> . . . . .	11.7	1.14	All intact cells, all gram-positive
Ground* $\left\{ \begin{array}{l} \text{in air} \\ \text{in vacuo} \end{array} \right.$ . . . . .	$\left\{ \begin{array}{l} 0 \\ 0.55 \end{array} \right.$	$\left\{ \begin{array}{l} 0 \\ 0.36 \end{array} \right.$	$\left\{ \begin{array}{l} \text{A few cells left, but mostly} \\ \text{gram-negative debris} \end{array} \right.$
Ground† with yeast extract $\left\{ \begin{array}{l} \text{in air.} \\ \text{in vacuo.} \end{array} \right.$ . . . . .	$\left\{ \begin{array}{l} 6.9 \\ 4.6 \end{array} \right.$	$\left\{ \begin{array}{l} 1.18 \\ 5.5 \end{array} \right.$	$\left\{ \begin{array}{l} \text{A few cells left, but mostly} \\ \text{gram-negative debris} \end{array} \right.$

\* Ground 18 hours in micro ball mill.

† Ground with an equal weight of yeast extract.

Typical data on the cell preparations obtained by this procedure are given in table 1. The original dried cell preparation oxidizes glycerol at a rapid rate, but glycerol phosphate very slowly, if at all. By grinding with yeast extract *in vacuo*, the rate of glycerol oxidation may be decreased to 40 per cent of the original value for dried cells, whereas the oxidation of glycerol phosphate is increased almost 5 times.

With most of the preparations employed it was necessary to have the yeast extract present during the grinding process. The addition of an equivalent quantity of yeast extract to the preparation after grinding did not restore the activity. Presumably, the yeast extract plays some part in the grinding process and is not merely a source of diffusible cofactors. An occasional preparation ground *in vacuo* without yeast extract could be activated by the addition of ATP (adenosine-5-triphosphate), as illustrated in figure 6. With such preparations glycerol addition does not result in oxygen uptake, but the further addition of



ATP causes oxidation (stopping at the toxic concentration of  $H_2O_2$ ). When ATP is added without glycerol, no oxidation occurs. The commercial glycerol phosphates are oxidized slowly by this preparation; the addition of muscle adenylic acid does not improve the respiration. This may be due to the inhibitory action of the inactive forms of glycerol phosphate on the oxidation of the active forms, since the addition of the mixed glycerol phosphates to cell preparations oxidizing glycerol results in an inhibition. The activation of glycerol oxidation by ATP (figure 6) indicates that the glycerol is converted into the natural glycerol phosphate before oxidation.

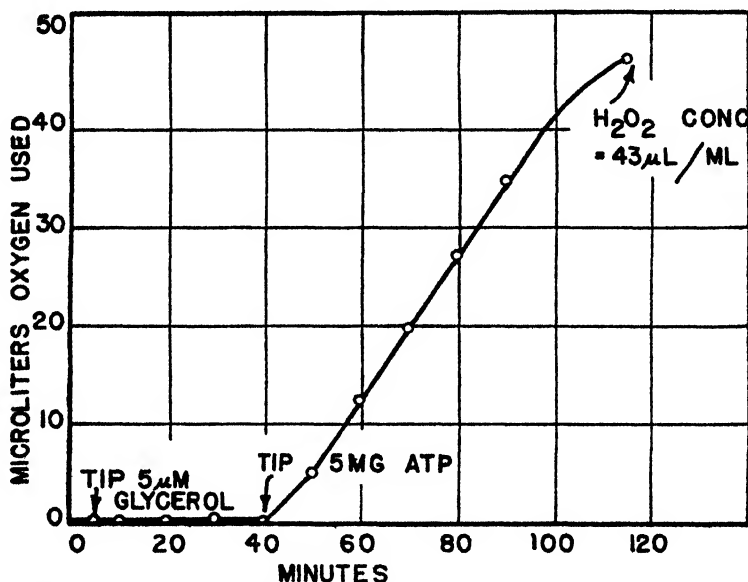


FIG. 6. ATP ACTIVATION OF GLYCEROL OXIDATION BY GROUND CELL PREPARATION

100 mg vacuum-dried cells (prep. 5) ground 18 hours in micro ball mill under vacuum and suspended in 3.3 ml  $M/15$  phosphate buffer pH 7.1.  
 0.5 ml (15 mg) suspension added per flask.  
 0.1 ml  $M/20$  glycerol (5  $\mu$  moles) and  
 0.5 ml Na ATP (10 mg/ml) added to side arm.  
 Side arms tipped as indicated, 37 C.

From these data the pathway of glycerol oxidation may be formulated with relative clarity. Glycerol reacts with ATP to form a glycerol phosphate, (presumably  $L(-)\alpha$ ; Baer and Fisher, 1939). This is the compound which is rapidly oxidized, probably to triose phosphate. The latter is converted into lactic acid through the same system followed by triose phosphates resulting from glucose fermentation. The glycerol oxidation system is thus part of a considerably more complex system of reactions.

It therefore becomes difficult to attribute the effect of an inhibitor to a reaction at a single point in this series. In the living cell, inhibition of any one of the reactions seems to result in the inactivation of the glycerol-oxidizing system. Therefore, it has not been possible to trap intermediates. Oxygen uptake on

glycerol is not inhibited by cyanide in the usual concentrations (i.e.,  $m/100$  to  $m/1,000$ ) but is inhibited by cyanide at levels of 0.03  $m$  (80% inhibition) and at 0.1  $m$  (100% inhibition). Iodoacetate (which was used in an attempt to block the system at the triose phosphate stage) completely inhibited the oxidation of glycerol at 0.001  $m$ . Sodium fluoride (in attempts to set up a similar block at the phosphoglyceric acid stage) showed 20 per cent inhibition of glycerol oxidation at 0.01  $m$ , 80 per cent at 0.03  $m$ , and 100 per cent at 0.075  $m$ . However, the action of fluoride appears to be on the glycerol system as such since pyruvate does not relieve the inhibition, as should be the case if transformations of the phosphoglyceric acids were inhibited.

Since the material reacting with oxygen appears to be a glycerol phosphate, it is of interest to compare this system with that of the animal. Two  $\alpha$ -glycerol-phosphate dehydrogenases have been reported: that of Euler *et al.* (1937) which is a coenzyme I system, and that of Green (1936) which is linked to cytochrome. If the enzyme system with which we are dealing is coenzyme I linked, it should be possible to obtain a conversion of glycerol to lactic acid under anaerobic conditions, providing pyruvate is supplied. However, when this was tried with resting cells, no acid was produced from glycerol nor from glycerol plus pyruvate under anaerobic conditions. Furthermore, with this strain (no. 24) other hydrogen acceptors (for example, fumarate) do not permit growth or acid production under anaerobic conditions. It appears that the oxidizing system is tied directly to oxygen, and we have been unable to find other substrates with which it will react. In a sense, therefore, this system resembles the enzyme of Green, but differs in its direct reaction with oxygen without the participation of the cytochrome system.

It is rather remarkable that a noncytochrome system is capable of the rapid reaction with oxygen exhibited during glycerol oxidation. When one considers that the preparations with which we are dealing are far from pure, and much inactive matter is contributing to the dry weight and the nitrogen, the capacity of the enzyme to utilize oxygen must be very great indeed. Although one would presume that the pure enzyme is a flavoprotein, it differs from known flavoproteins in the rapidity with which it reacts with oxygen. The rate of oxidation of glycerol is not increased by the use of pure oxygen instead of air nor by the addition of methylene blue.

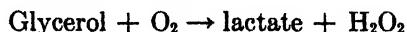
The dried cells may be treated with ammonium sulfate at pH 3 to 4 (as is used in the separation of the flavin from the protein with Warburg and Christian's old yellow enzyme), or subjected to the procedures used by Lipmann (1939) and by Straub (1939) for separating the flavin component from their preparations, without removing activity from the cell particles. The separation used on the amino acid oxidase (pH 5, 1/2 saturated  $(NH_4)_2SO_4$ ) completely inactivates the glycerol system (although it is less drastic than the ones previously mentioned), and we have been unable to restore the activity. Also, the relation between the activity of the cells on glycerol and the flavin content of the medium is not a direct one. Hence, detailed knowledge of the nature of the enzymes involved in glycerol oxidation must await their isolation.

The evidence presented to show the course of glycerol oxidation also bears upon the functional nature of this respiration. Certainly the glycerol oxidation provides energy to the cell since this streptococcus will grow well when glycerol is supplied aerobically, whereas very little growth occurs in the base medium (Gunsalus and Sherman, 1943). However, the respiration need not yield energy directly to the cell, for, after glycerol is converted into triose phosphate, the latter can be fermented to lactate and provide energy. The powerful respiratory system operating on glycerol may, therefore, be merely a means which the cell employs to remove two extra hydrogens, and the cell may derive its energy, not from the respiration as such, but by fermenting the product of that respiration. There is no critical evidence at present that the cell derives energy from the respiration as such.

The uptake of exactly one mole of oxygen per mole of glycerol suggests that there is no very direct pathway to oxygen from the coenzyme I stage, and the low oxidation of glucose and lactate as shown in figure 1 strengthens this conclusion. However, in working with this organism over the past three years, we have noted a considerable variation in the ability of different suspensions to oxidize glucose and lactate. A similar variability was noted by Barron and Jacobs (1938). Suspensions which oxidize glucose and lactate at the more rapid rates (or preparations therefrom, for example as in figure 4) take up slightly more than one  $O_2$  per glycerol—the extra oxygen uptake being correlated with lactate or glucose oxidation which presumably proceeds through reduced coenzyme I. The data in figure 4 illustrate the type of result obtained. While this variability has not yet been traced to its cause, its occurrence does not influence the conclusions obtained with the glycerol system.

#### SUMMARY

Glycerol is oxidized by *Streptococcus faecalis*, strain 24, at a rapid rate ( $Q_{O_2}(N) = 400-700$ ), the over-all reaction being:



The pathway of this oxidation involves phosphorylation. The glycerol is phosphorylated (with ATP) to glycerol phosphate, which is oxidized to triose phosphate. The latter is converted into lactic acid.

The oxidation is inhibited by the accumulation of  $H_2O_2$  to a concentration of 0.002 molar.

Various properties of the enzyme system are described.

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# THE VALUE OF BACTERIOPHAGE IN CLASSIFYING CERTAIN SOIL BACTERIA<sup>1</sup>

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## INTRODUCTION

One of the most serious difficulties confronted by soil bacteriologists in trying to draw practical conclusions from their study of microorganisms in soil has been their inability to distinguish the various species one from another. Certain species (notably the sporeformers and rapidly liquefying nonsporeformers) can be fairly well recognized and classified, but they are not the most abundant nor presumably the most active microorganisms in soil. Examination of soil either by the plate method or the microscopic method shows the presence of much more numerous (and presumably important) bacteria with characteristics so indefinite in nature that it is difficult to determine whether the group includes hundreds of species or perhaps only one or two. Under such circumstances progress in the study of soil bacteria has naturally been slow. How much progress in the control of insect pests would have been made if entomologists were unable to tell one kind of beetle from another?

Under such circumstances soil bacteriologists have, like drowning men, grasped at every straw in sight, and most of them have proved very frail supports. A study of morphology has yielded some progress, to be sure, allowing classification into groups showing the characteristics of *Corynebacterium*, *Mycobacterium*, "coccus-forming rods," or simple rods. This has never taken the classification far, however, as evidenced by the fact that whereas Conn (1928) considered the "coccus-forming rods" as being probably all one species, *Bacterium globiforme*, Lochhead and Taylor (1938) refer to the *Bacterium globiforme* group, which they regard as comprising numerous species all having the type of morphology in question. Even less progress has been made from the use of physiological characters; sugar fermentations, nitrate reduction, and numerous other similar features have been investigated, and all more or less abandoned, either as giving only negative results or as proving too variable, in this group, for reliable use in classification. The authors of the present paper wish to point out that another attack can be made through the use of bacteriophage. It is recognized that a race of bacteriophage developing in a bacterial culture is to a certain extent specific for the species of bacteria in association with which it has developed. It is obvious that if this specificity were absolute, it would furnish a means of distinguishing species of bacteria from one another, even though no differences could be established by ordinary cultural methods.

Ever since the specificity of bacteriophage was first observed, its possible value

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as an aid in bacterial classification has been under consideration. Among the first to investigate this possibility was Laird (1933) who made a study of various strains of certain *Rhizobium* spp. and their susceptibility to certain types of phage. Laird concluded that there was too great variation in susceptibility for this method of attack to be promising.

On the other hand, Evans (1934, 1936, 1940) has found a certain degree of correlation between phage resistance and ability of certain streptococci to produce septic sore throat. Keogh, Simmons, and Anderson (1938) have found susceptibility to phage of some use in classifying diphtheria strains. Levine and Frisch (1935) have observed some correlation in the case of *Salmonella* spp., and Miller (1937) found the method to have value in the identification of the dysentery organism.

The general conclusion since the work of Laird has been that the method is impractical in the case of legume nodule bacteria. Demolon and Dunez (1938), for example, found various races of phage nonspecific in their action on different strains of the organisms. In the light of this previous work it would have seemed hopeless to attack the problem further except for the improved methods introduced by Campbell and Hofer (1943). Their observations indicate that Laird's medium does not support vigorous growth of the organisms and rarely becomes entirely clear, even when phage has been added and lysis has occurred. This would easily bring about irregularity in results of the type which Laird observed and which he thought indicated nonspecificity. As a result, further investigations of this matter have been made, using Campbell and Hofer's medium and employing the more refined technique which modern studies of bacteriophage have made appear desirable.

The technique is, to be sure, rather complicated and calls for close attention to detail; but it is no more difficult or involved than the immunological methods that have yielded such promising results in the study of other groups of bacteria.

#### METHODS

*Preparation of bacteriophage.* The first step was to select a soil in which bacteriophage effective against the organism under investigation was believed to be present. In the case of common soil bacteria (like *Agrobacterium radiobacter* and the *Bacterium globiforme* group) the choice of soil made little difference; but when bacteriophage for one of the legume organisms was being sought, a soil was selected on which the legume in question had been growing.

Phage filtrates active against the various cultures were obtained by inoculating 1 ml of a heavy suspension of a 24-hour culture of each into 50 ml of Campbell and Hofer's (1943) sauerkraut glycerophosphate medium, then mixing with 100 g of the selected soil and incubating 20 to 24 hours at 30 C. The infusion was then filtered, first through two thicknesses of medium-speed filter paper until clear, then through a sterile Berkfeld N or W filter candle. The sterile filtrate thus obtained was designated filtrate A.

The filtrate A in each case was tested for lytic activity by the dilution method. In the first test, 1 ml of the filtrate was added to 9 ml of the medium, and inocu-

lated with the organism with which the soil had been inoculated by adding 1 to 5 drops (or in case of slow-growing cultures, 1 ml) of a 24-hour culture. (Clear tubes of uniform diameter were used throughout.) The tube was incubated at 30 C, and observations on lysis were ordinarily made after 24 hours; sometimes this was checked by observations on a second tube at the end of 48 hours. (In this tube filtrate A had been diluted 10 times, and presumably any ingredient thereof which was incapable of multiplication remained diluted to that extent; but the incubation allowed multiplication of any bacteriophage present to which the organism inoculated into it was susceptible.) This diluted and incubated filtrate was treated in one of three ways:

- 1) If slight lysis appeared after the first incubation, the contents of the tube were filtered, and dilutions of 10 and 1,000 times made in the sauerkraut glycerophosphate medium. If only partial lysis was observed in the inoculated 1,000 dilution after 24 hours of incubation, it was filtered, and again dilutions of 10 and 1,000 times were made, inoculated, and incubated. Serial transfers from the 1,000 dilution were continued in this same manner, by filtering, making dilutions, inoculating, and incubating, until complete lysis occurred in the 1,000 dilution in 24 hours.

- 2) If complete lysis occurred after the first 24-hour incubation, the contents of the tube were assumed to be practically sterile, and further tests were made without filtration; in this case a dilution of 1/1,000 was made directly and observations were made for lysis after 24-hour incubation; if no lysis was observed in the first such tubes, further dilution was made until after 24-hour incubation the 1/1,000 dilution did show complete lysis.

- 3) If no lysis, or only questionable lysis, occurred after the first incubation, the contents of the tube were filtered and 2 ml of the filtrate were added to a tube containing 9 ml of the sterile medium, inoculated, and incubated. No further work was carried on with the contents of the latter tube if no lysis occurred after 48 hours. If there was evidence of lysis, however, the usual procedure of filtering, diluting 10 and 1,000 times, inoculating, and incubating was continued until the 1/1,000 dilution showed complete lysis.

If this method was not enough to permit development of bacteriophage from some particular culture, greater concentration of the phage was secured by the following method: filtrate A was not diluted immediately, but 9 ml of it were poured into a sterile test tube and inoculated with the organism for which a bacteriophage was being sought. After 24 hours it was filtered and the entire filtrate (about 5 ml) brought up to 9 ml by addition of more of the medium. This was then inoculated and incubated, and the process continued until complete lysis was observed in 24 hours. Then the usual series of 1/10 and 1/1,000 dilutions was made, inoculated, and incubated, with serial transfers every 24 hours, from whichever tube showed most lysis, until complete lysis was observed in the 1/1,000 dilution. If no lysis was observed after about 10 transfers, the filtrate A in question was discarded as having no bacteriophage for the organism for which it was being sought.

Six successive 1/1,000 dilutions were made of these lysed cultures, in which



filtrate A had already been diluted at least 1,000 times. (This resulted in a final dilution of  $10^{-21}$  or more of any substance in filtrate A which had not been multiplying during the process.) They were not filtered between transfers unless for some reason the tubes were not completely cleared. In this manner extraneous material from the soil was diluted out and the titer of the bacteriophage increased simultaneously.

Larger quantities of the phage were prepared by adding a tube of a 1/1,000 dilution which had been lysed for 24 hours and 1 ml of the homologous culture to 50 or 60 ml of the medium in a flask. This was incubated overnight and filtered, and designated filtrate B.

The lytic activity of filtrate B was tested by a slightly different dilution method. It was diluted in water blanks, or in tubes of sterile medium, of several different dilutions; 1 ml of each dilution and 1 to 5 drops of a 24-hour culture of the homologous strain were added to 9 ml of the medium and incubated at 30 C. The highest dilution showing lysis in 48 hours was taken as the critical dilution. As an arbitrary standard, it was considered essential that lysis should occur with a dilution of  $10^{-7}$ . Any filtrates failing to show lysis up to this dilution were reinoculated with 1 ml of a 24-hour culture, incubated overnight, and again filtered. These filtrates (C) were again tested. This process was repeated until the titer was brought up to the desired point. Phage filtrates (either B or C, according to initial strength) were prepared in this way from various strains of the organisms investigated in this work.

*Testing for cross lysis.* To test for cross lysis of the various cultures under investigation, 1 ml of each of their phage filtrates (either B or C) was added to 9 ml of a 24-hour culture of the organism to be tested. Controls, inoculated but containing no phage, were used as the standard of comparison in making observations for lysis. The amount of lysis was recorded after 24 and 48 hours of incubation at 30 C. A control of the sterility of the phage filtrate was run by adding 1 ml thereof to 9 ml of the uninoculated medium and incubating.

## RESULTS

### *Preliminary experiment: comparison of the pea organism with other nonsporeformers from soil*

The first preliminary test was made to observe whether cross lysis occurred between some *Rhizobium* species, the very similar *Agrobacterium radiobacter*, and certain cultures belonging to the *Bacterium globiforme* group. Although most of the tests included in this preliminary work were repeated in the more intensive work which followed, a summary of the preliminary experiment is given here in table 1 because this was the only time in the course of the work that these three groups of organisms were tested simultaneously. The cultures used in this preliminary test were as follows:

*Agrobacterium radiobacter:* Cultures S<sub>188</sub> and S<sub>88</sub>, from N. R. Smith of the U. S. Department of Agriculture, the latter an old strain brought to this country by F. Löhnis; cultures 1000, R<sub>1-1a</sub>, and R<sub>3</sub>SC<sub>1</sub> from the University of Wisconsin,

the last one a single cell isolation; culture ISC<sub>1</sub> from the Department of Bacteriology at the Iowa State College.

*Bacterium globiforme* group: A culture labeled "Bact. glob." which has been regarded as typical of the species and has been distributed from this laboratory under the specific name in question; six other stock cultures which have been maintained in this laboratory for ten years or so and which have all been found

TABLE 1

Preliminary tests for cross lysis: *Agrobacterium radiobacter*, the *Bacterium globiforme* group, and the pea nodule organism\*

BACTERIOPHAGE	CULTURES															
	<i>A. radiobacter</i>						<i>B. globiforme</i> group						<i>Rhizobium</i> ( <i>pea</i> )			
	RSC <sub>1</sub>	S <sub>188</sub>	ISC <sub>1</sub>	1000	R <sub>1-18</sub>	S <sub>38</sub>	Bact. glob.	ECk <sub>2</sub>	CSk <sub>2</sub>	AS2k <sub>2</sub>	OSC3k <sub>2</sub>	BCK <sub>4</sub>	BCK <sub>2</sub>	B2	B6	B12
<i>A. radiobacter</i>																
R <sub>3</sub> SC <sub>1</sub> .....	++	++	++	++	++	++	-	-	-	-	-	-	-	-	-	-
S <sub>188</sub> .....	++	++	++	++	++	++	-	-	-	-	-	-	-	-	-	-
ISC <sub>1</sub> ....	++	-	++	++	++	++	-	-	-	-	-	-	-	-	-	-
1000.....	++	-	++	++	++	++	-	-	-	-	-	-	-	-	-	-
R <sub>1-18</sub> .....	++	++	++	++	++	++	-	-	-	-	-	-	-	-	-	-
S <sub>38</sub> .....	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-
<i>B. globiforme</i> group																
Bact. glob....	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-
ECk <sub>2</sub> .....	-	-	-	-	-	-	-	++	++	++	++	-	-	-	-	-
CSk <sub>2</sub> .....	++	-	++	++	++	-	++	++	++	++	++	-	-	-	-	-
AS2k <sub>2</sub> .....	-	-	-	-	-	-	-	++	++	++	++	-	-	-	-	-
OSC3k <sub>2</sub> .....	-	-	-	-	-	-	-	-	++	++	++	-	-	-	-	-
BCK <sub>4</sub> .....	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-
BCK <sub>2</sub> .....	-	-	-	-	-	-	++	-	-	-	-	-	++	-	-	-
<i>Rhizobium</i> ( <i>pea</i> )																
B2.....	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	++
B6.....	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	++
B12.....	-	-	-	-	-	-	++	-	-	-	-	-	-	++	++	++

\* The symbols used in this table have the following significance:

++ Complete lysis in 48 hours.

- No lysis in 48 hours.

to show the typical morphology of *Bacterium globiforme*, i.e., gram-negative rods in 24-hour culture, gram-positive cocci after 2 or 3 days.

*Rhizobium leguminosarum*: Three cultures isolated from plates made in this laboratory from a commercial culture sold for the inoculation of peas.

The most important fact brought out by table 1 is that although a high degree of cross lysis was observed between the individual cultures in each of the three

groups, there was almost none between the groups. In fact, there were only two phage filtrates that lysed any culture or cultures outside their own group. These were so few they might well be caused by impure phage preparations, and their occurrence seemed to indicate the need of a more rigorous technique. Accordingly the work was continued with more effort to run the bacteriophage through enough serial transfers to assure its purity.

*Agrobacterium radiobacter, Bacterium globiforme, and miscellaneous soil forms*

After the preliminary investigation, it seemed best to divide the cultures into two lots for study. One lot consisted of the legume nodule bacteria, and results with them are discussed in a later section; the other lot of cultures, which is taken up in the present section, consisted of 7 strains of *Agrobacterium radiobacter* (all but one the same as in the preliminary work); 14 cultures of organisms showing the typical *Bacterium globiforme* morphology, including the stock cultures used in the preliminary experiment and others freshly isolated from soil; and 7 miscellaneous small nonsporeforming bacteria recently isolated from soil.

This work was carried out during a period of three or four years; two different laboratory workers handled the cultures, obtained bacteriophage from them, and made the tests for lysis. Tests for cross lysis were always repeated once and sometimes two or three times by each of the two workers. Results did not always agree on repetition, but there was sufficient agreement so that interesting conclusions can be drawn. A summary of all the data obtained with this group of cultures is given in table 2.

The results indicated for *Agrobacterium radiobacter*, in the upper left-hand block of the table, are essentially the same as those in table 1, but represent a good many more tests. In general, cross lysis occurred among the various strains of this species. About the only exception was culture S<sub>188</sub>, which was lysed by only three of the phage preparations. It should be remarked that by the time the second worker (R) took up the investigation it no longer proved possible to get this culture to develop bacteriophage; presumably it was highly phage-resistant from the start and was getting more so during the progress of the investigation. Culture S<sub>36</sub>, on the other hand, lysed not only its homologous strain when tested by B, but acted upon four of the other cultures when tested by R. Another striking fact, brought out by the table, is that no culture of this organism was lysed by the phage from any of the other bacteria included in this series of tests except that from culture CSK<sub>1</sub>, which did lyse four of the *A. radiobacter* strains in the hands of the first laboratory worker (B) but not when the tests were repeated by the second (R). It is equally striking that the phage filtrates obtained from this species failed to lyse any of the other organisms.

It should be added that an experiment was carried on to show whether these tests confirm the differences known to exist between *A. radiobacter* and *Rhizobium*. Phage from one culture of the former (R<sub>1</sub>SC<sub>1</sub>) was tested on 17 cultures of various *Rhizobium* species; and all the *A. radiobacter* cultures were tested with bacteriophage from lima bean and soy bean cultures. No lysis was observed in any of these instances.

The results obtained with the "globiformelike" cultures are similar to those in table 1; but the larger number of strains included and the frequent repetitions of the tests make the results more significant. Arranged as they are in the table, it will be seen that there is a group of six cultures that shows as good cross lysis as do the *A. radiobacter* strains; it is not difficult to infer that they are all the same species. Of the other eight cultures, three (RJ4c, d, and e) cross-lyse perfectly; one culture (BCK<sub>4</sub>) shows no cross lysis with any other culture; the other four show a little cross lysis with scattered strains, but not consistently. From this one can infer either that one species occurs commonly in the soils from which the isolations were made and that the other cultures represent separate species, or that only one species is represented and that this test of cross lysis is not satisfactory for its classification. On the whole, the first of these two assumptions seems the more probable. Somewhat unfortunately, the particular strain that has been distributed from the writers' laboratory under the name of *Bacterium globiforme* is not one of these that cross-lyses with any other strain.

The three cultures designated RJ4c, d, and e, call for special comment. They are all subcultures from a single original isolate which showed such varied morphology as to seem impure. It was plated out, and these three subcultures showed such differences in 24-hour cultures that they were believed to be separate species: one was a long rod, one so short as to be regarded a coccus, and the third was intermediate in length; all three, however, were coccuslike when two or three days old. It is clear from table 2 that all three develop identical races of bacteriophage; so presumably they are all the same species and the difference in morphology when 24 hours old merely indicates differences in their rate of going through the life cycle. The fact that the bacteriophage test brings out this apparent identity among them speaks well for the value of the test when applied to such organisms as these.

The last block of cultures listed in table 2 consisted of six short rod forms and one coccus that did not show the typical "globiformelike" morphology. These cultures were all fresh isolations, not yet completely studied; hence, little is known as to their identity. They are known, however, to be nonsporeformers of the same general type, biochemically, as *Bacterium globiforme*; their characteristics, in other words, are largely negative, and too indefinite to permit good characterization into species. It was once thought possible that all such organisms might comprise a single species.

The results of this bacteriophage test hardly confirm such an assumption. A glance at the lower right-hand block of table 2 is enough to show that there was no instance of cross lysis among these cultures except in the case of RWi4b and RWi4c. These two cultures (like RJ4c, d, and e) were subcultures from colonies obtained on plating out an isolate that was thought to be impure; they are therefore regarded as unquestionably identical.

It is clear, therefore, that bacteriophage tests bring out differences between these various strains of soil bacteria that are not evident by ordinary cultural or biochemical tests. Whether the cultures from which distinct races of phage are obtained are actually separate species is still an open question. The chief







[illegible]

\* The symbols used in this table have the following significance:

†† Complete lysis in 48 hours.

**+ Partial lysis in 48 hours.**

— No lysis in 48 hours.

Disagreement: B's results ++, R's results -.

Disagreement: B's results ++, R's results -.  
Disagreement: B's results -, R's results ++.

† These two phage filtrates were tested on all seven *A. radiobacter* cultures listed in table 2; no lysis occurred.



reason why one can only speculate on this subject is that in this group of bacteria too few other tests are known that can be used to correlate with the results obtained from the bacteriophage tests. For this reason, special interest attaches to the cultures discussed in the next section in regard to which there is a definite characteristic with which such results can be correlated—namely, ability to produce nodules on certain species of legumes.

### *Cultures of Rhizobium species*

The preliminary work (table 1) included studies with three cultures of the pea nodule organism, which is the cross-inoculation group to which the specific name *Rhizobium leguminosarum* is ordinarily regarded as applying exclusively. The three strains cross-lysed perfectly with each other and, except that one of them lysed a *Bacterium globiforme* culture (which might easily have been due to an impurity in the phage filtrate), there was no cross lysis with any of the other cultures included in that series of tests.

This made it seem worth while to extend the work to include various strains of other cross-inoculation groups. The following cultures were secured:

For clover: 200, 201, 202, 205, 207, and 209, obtained from the University of Wisconsin.

For pea: 301, 303, 304, 310, 311, and 313, from the University of Wisconsin; B4, an isolate from a commercial inoculant.

For bean: 400, 403, 407, and 403sc, from the University of Wisconsin, the last named having been obtained by single cell isolation from 403.

For alfalfa: 100, 110, 116, 123, 124, 125, 128, and 32b, from the University of Wisconsin.

For soy bean: 504, Soy 14, from the University of Wisconsin.

For lima bean: 602, from the University of Wisconsin; R1a; a pure strain from a commercial inoculant company.

After the tests with these organisms had been carried on by the first worker (B), they were repeated a year or so later by R, with an entirely new set of phage filtrates developed independently from soil. After several repetitions of the cross-lysis tests, table 3 was prepared to summarize all the results.

The most striking fact brought out by this table is that the clover, bean, and pea organisms in general cross-lyse, whereas there is only very occasional cross lysis between any of the other cross-inoculation groups. Cross lysis among all the strains of the clover-pea-bean group is not perfect; but it is clear from a glance at the table that in those instances in which any given phage filtrate has failed to lyse, one of the cultures is just as apt to occur with a culture isolated from the same legume as with one of the other legumes of the group.

The bacteria from soy bean, lima bean, and miscellaneous legumes did not yield bacteriophage so readily—largely, it is thought, because these organisms do not grow so well in the medium employed in this work. Such data as have been secured are included in the table; they are incomplete, but at least do indicate that none of these cultures are lysed by bacteriophage from the clover, pea, bean, or alfalfa organisms.

What is the significance of these results? They do not necessarily mean that the clover-pea-bean group is all one species. What they do show is that essentially identical phage filtrates are produced from the 16 cultures studied that had been obtained from these three legumes. This certainly indicates that cultures from these legumes are very much more closely related to one another than they are to those from alfalfa, soy bean, and lima bean. This observation correlates nicely with results of morphological and cultural studies of the organisms; it is well known to students of *Rhizobium* that the pea, bean, and clover organisms are practically indistinguishable except by inoculation tests, and there is belief in some quarters that cross inoculation between these groups may be common. The alfalfa organism, on the other hand, has distinctive characteristics, other than its association with legumes, which allow it to be recognized. The soy bean and lima bean organisms are still different.

This correlation, if further studies bear it out, may well be enough to show that the clover-pea-bean group represents one species; if so, one would have to recognize it by the oldest (and as it happens most appropriate) name, *Rhizobium leguminosarum*, and the names *Rhizobium phaseoli* and *Rhizobium trifolii* would have to be dropped. Whether or not such a regrouping of the organisms is justified, it is clear that the method of comparison employed in this paper is more promising than the conclusions of Laird (1933) indicated.

One point of special interest brought out by these studies is that the bacteriophage test correlates with the cross-inoculation grouping of the bacteria. The groups established are larger than the cross-inoculation groups, but the lines of division brought out by the two sets of criteria are parallel. This is the opposite from what has been found when efforts have been made to classify legume nodule bacteria by serological methods. The agglutination test, for instance, was found by Stevens (1923) to show from two to four agglutination groups in each of seven cross-inoculation groups which he studied. This fact seems to indicate that the groups established by means of bacteriophage are of more significance than those indicated by means of agglutination.

#### SUMMARY

In spite of the discouraging reports of previous investigators, an effort was made to employ the specificity of bacteriophage as a criterion in classifying legume nodule organisms and certain other soil bacteria. It was found that by using methods of cultivating phage which have been proposed by Campbell and Hofer (1943), encouraging results can be obtained.

It was shown that phage filtrates, developed from each of six strains of *Agrobacterium radiobacter*, were usually able to lyse completely all of the six strains; whereas in the case of a seventh strain that evidently yielded a phage of lower potency, the phage filtrate gave variable results. These phage preparations practically never lysed cultures of any *Rhizobium* species or of the miscellaneous soil organisms studied; the instances of such cross lysis were so few that it was easy to explain them as probably due to contamination with some bacteriophage other than the type that had been sought.

Of fourteen cultures showing the type of morphology typical of *Bacterium globiforme*, six showed almost perfect cross lysis with one another; the other eight cultures showed little cross lysis, several of them apparently developing their own specific type of bacteriophage. It happened that three of these six cultures were subcultures from one original strain, and these three *did* cross-lyse, a fact which indicates that the method is a fairly satisfactory means of showing identity of strains. These results indicate that there may be numerous species in soil showing the "globiformelike" morphology; a fact which seems to justify Loch-head in speaking of the "*Bacterium globiforme* group."

Seven miscellaneous cultures of nonsporeformers from soil were studied, all of which showed the type of colony characteristic of *Bacterium globiforme*; one was a coccus, the others small short rods. The phage filtrates developed from these miscellaneous forms were capable of lysing only their homologous strains. The only exception to this was in the case of two subcultures from a single original isolate—another exception to prove the rule! From this it seemed evident that each of these isolations from soil represented a distinct species, although some of them were indistinguishable from one another by ordinary bacteriological tests.

Thirty-three cultures of *Rhizobium* were studied, which belonged to six well-known cross-inoculation groups, as well as three cultures from three less common legumes. The study included six strains from clover, seven from pea, and four from bean, all of which showed almost complete cross lysis, without regard to which of the three legumes they were derived from; but so rarely did any of these phage filtrates lyse other legumes that when such activity was observed contamination of the phage was immediately suspected. Eight strains of the alfalfa organism were studied and found to show a high degree of cross lysis among themselves, but not with cultures from other legumes. In the case of soy bean and lima bean cultures, it proved more difficult to obtain potent bacteriophage; but one race of phage active against each of these two species of *Rhizobium* was obtained, and neither of them proved able to lyse cultures from any type of legume other than that associated with the culture from which it was derived.

These results indicate that the clover, pea, and bean organisms all develop identical races of phage. Whether this means that they are all of one species is still an open question; but it must be remarked that cultures of these three cross-inoculation groups are well known to be almost identical in morphology and cultural characteristics. It is generally admitted by students of the group that these three are much more closely related to one another than to the alfalfa organism or to those from soy bean or lima bean. Hence the results with bacteriophage bear out previous findings obtained by other methods, whether or not they settle the question of specificity in this group.

It is, therefore, concluded that the bacteriophage method for classifying closely related types of bacteria has its value. Although, used alone, the method probably cannot separate one species from another, it is valuable as a supplementary test in cases of doubtful specificity.

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# ON THE MICROSCOPIC METHODS OF MEASURING THE DIMENSIONS OF THE BACTERIAL CELL

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It has been shown by the author (Knaysi, 1941) that the dimensions of cells measured on smears dried, fixed, and stained by the ordinary methods are considerably below those of cells in the living state and represent the dimensions of the shrunken protoplasm. More recently (Knaysi, 1944) the various methods of microscopic measurements were discussed, and it was concluded that values approaching true cell dimensions can be obtained either by measuring the living cells or in smears especially stained to show the cell wall. Measurements from electron micrographs were considered satisfactory when the cell wall is visible. It was also stated: "Figures obtained from negative smears are too high because they include also the slime layer usually surrounding the cell. In the case of capsulated organisms, the relative error may be tremendous. There is also an additional error due to the retraction, upon drying, of the colored film in which the bacteria are imbedded."

Working with *Bacillus megatherium*, Dubin and Sharp (1944) compared the dimensions of the same air-dried, unstained cells in photo and electron micrographs. They concluded: "Images in the electron micrographs indicate plainly the presence of two structures constituting the bacterial cell, an inner dense substance and an outer less dense substance. This outer substance of the bacterial cell is invisible in the light micrographs. . . . There is no significant difference between measurements of the size of unstained bacteria dried in air and photographed with the light microscope and measurements of the comparable portion (inner dense substance) of the identical bacteria later photographed in the electron microscope." Dubin and Sharp noted the agreement between their results and those of the author. However, their study of cell dimensions on negative preparations (Benian's method) led them to the conclusion that they were "less than those of the total bacterial substance seen in the electron micrographs," which is "not in agreement with Knaysi's observation that in negative-staining preparations the figures are even slightly higher than the true size because of the retraction of the colloidal film upon drying."

This *apparent* disagreement between the results of Dubin and Sharp and those of the author made it desirable to reinvestigate this elementary, but important, problem of bacterial morphology, the problem of correct measuring of cell dimensions.

## ORGANISMS AND METHODS

The organisms studied were strain C<sub>2</sub> of *Bacillus mycoides* and strain C<sub>2</sub> of *Bacillus cereus* previously classified in this laboratory by Lamanna (1940). We

preferred these two organisms to *Bacillus megatherium*, the cells of which have a tendency to curve or coil.

Smears for cell wall and negative staining were prepared from the same suspension of cells in distilled water from a 6-hour-old culture on beef infusion agar slants at 33 C. Living cells were studied in microcultures of the same strains on the same medium; measurements were made on photomicrographs prepared as previously described (Knaysi, 1940). In view of the well-known wide variation in cell length, only the width of the cells was measured. This dimension is nearly the same for young cylindrical cells growing in nearly the same environment (figures 1 and 6). The width at the median part of the photographic image of a given cell was measured with dividers, and the value in microns was obtained with the help of a scale similar to that previously used (Knaysi, 1929). In order to make this time-saving and very accurate scale better known, we are reproducing it, with directions for its use and construction, in figure 8.

#### RESULTS AND DISCUSSION

The results of the present study are illustrated in figures 1-7.

*Living cells.* The cells of figure 1 represent a microculture of *Bacillus mycoides* in the early stages of development. They are not all the progeny of a single cell and may be taken to represent what one may encounter in an actively growing slant culture. Nevertheless, one can easily see that variations in width are relatively slight. Careful measurement of twenty cells similar to those of figure 1 shows that, regardless of the errors of measurement, the width of the various cells falls within 1.13 and 1.20  $\mu$ , with an average of 1.17  $\mu$ . Among the progeny of a single cell, the differences are even smaller; this is shown in figure 6 for *Bacillus cereus* (see also Knaysi, 1941, and plates III and IV, 1944), where the width may be considered constant and equal to 1.68  $\mu$ .

*Cell wall method.* Figure 2 shows young cells of *Bacillus mycoides* stained by the author's cell wall method (Knaysi, 1941). Measurement of 14 cells similar to those of figure 2 gave a range of 1.07 to 1.25 and an average of 1.16  $\mu$  (compared with 1.17  $\mu$  for living cells). The fluctuation here is 0.18  $\mu$ , about 2.5 times greater than that for the living cells. However, part of this fluctuation is undoubtedly due to the fact that the cells on a slant culture are not growing in as uniform an environment as that of a microscopic field of a plane microculture; furthermore, there are probably differences in the degree of shrinkage and dye adsorption between different cells. It must also be emphasized that those differences are brought out here by the accuracy of the methods of measurement and would probably pass unnoticed by the usual methods used by the bacteriologist. This method gives for *Bacillus cereus* (figure 7) a width of 1.6  $\mu$ .

Of interest is a comparison between figures 5 and 7. In both figures the cells came from the same suspension. The cells of figure 5, however, were stained with Meyer's methylene blue (air-dried and mounted in oil) and obviously correspond to the central, cylindrical mass of shrunken cytoplasm of figure 7. The width of this mass in figure 5 varies from 0.50 to 0.75  $\mu$  with an average of 0.61  $\mu$ ; it compares with the width of the central mass of figure 7 and amounts to about

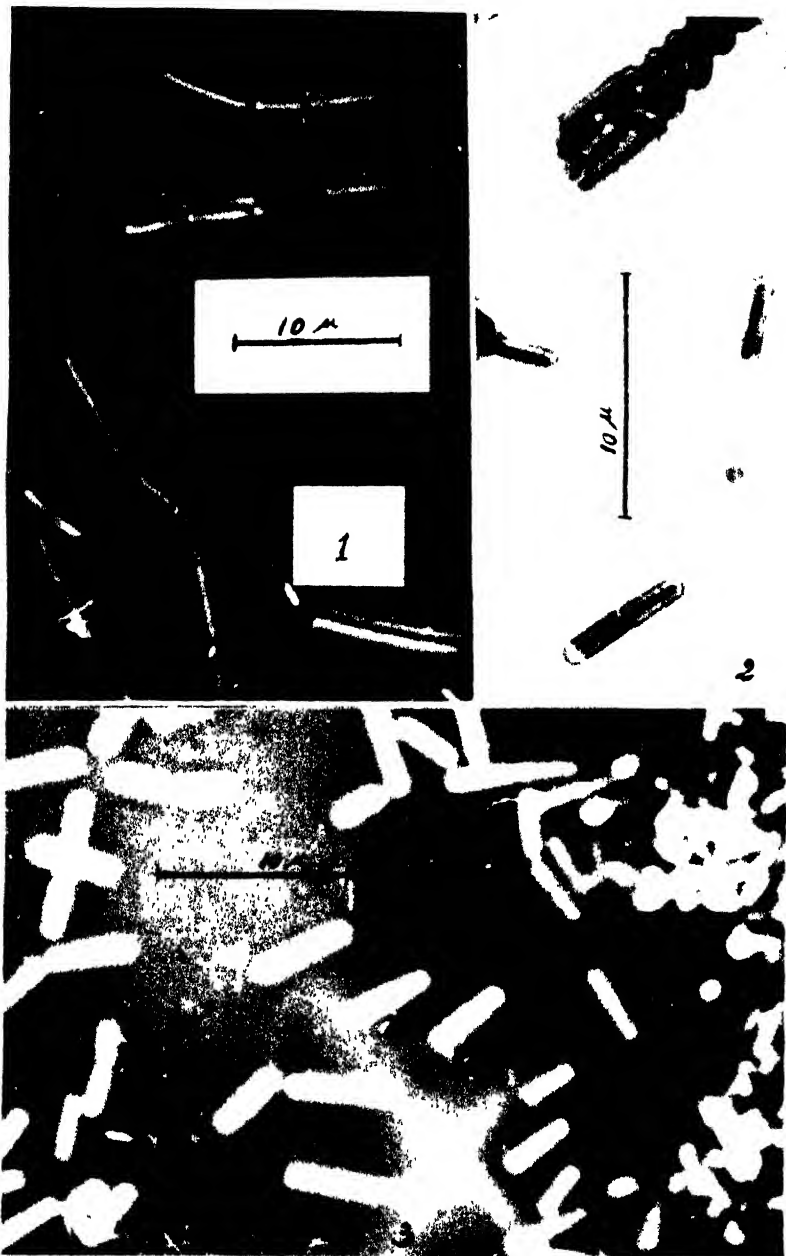


FIG. 1. *BACILLUS MYCOIDES*, STRAIN C<sub>2</sub>

Cells in a 3- to 4-hour-old microculture at 31 to 32.5 C on meat infusion agar (pH 7), photographed in dark field.

FIG. 2. *BACILLUS MYCOIDES*, STRAIN C<sub>2</sub>

Cells from a 6-hour-old slant culture at 33 C on the medium: meat infusion (half concentration) + 0.5% tryptone + 0.5% glucose + 1.5% agar; pH 7. Cell wall method. Photographed using Wratten filters B58 + E22.

FIG. 3. *BACILLUS MYCOIDES*

Strain and culture as in figure 2. Negative preparation with water-soluble nigrosine. Photographed using Wratten filter B58.



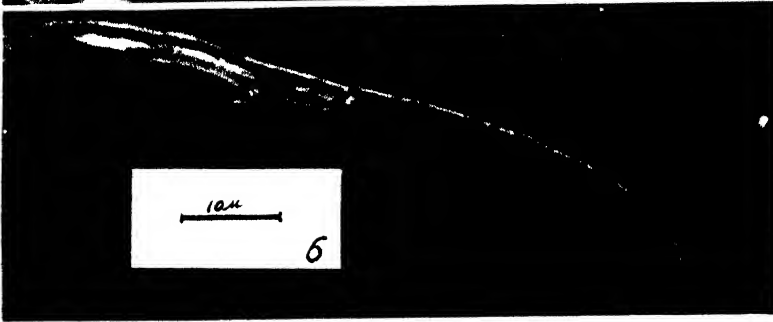


FIG. 4. *BACILLUS MYCOIDES* (SEE CAPTION FOR FIGURE 3)

FIG. 5. *BACILLUS CEREUS*, STRAIN  $C_3$

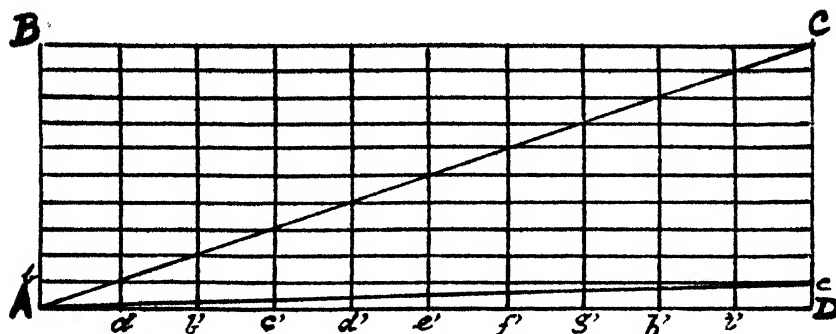
Cells from a 6-hour-old slant culture in meat infusion agar (pH 7) at 33 C. Stained with methylene blue and mounted in oil. Photographed using Wratten filter A25.

FIG. 6. *BACILLUS CEREUS*, STRAIN  $C_3$

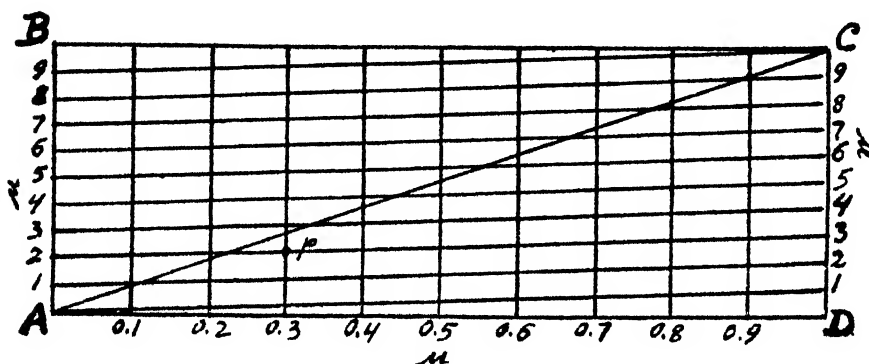
Cells in a 7-hour-old culture at 31 to 32.5 C in meat infusion agar (pH 7). Photographed in dark field.

FIG. 7. *BACILLUS CEREUS*

Strain and culture as in figure 5. Cell wall method. Photographed using Wratten filters B58 + E22.



I



II

FIG. 8. In part I, Side  $\overline{AB} = \overline{DC}$  of rectangle  $ABCD$  is equivalent to  $10\ \mu$ . Sides  $\overline{AD}$  and  $\overline{BC}$  are divided into 10 equal parts, and the points of division are joined by the perpendiculars  $a', b' \dots i'$ . The diagonal  $\overline{AC}$ , having a constant slope, intercepts on each of the perpendiculars  $a', b', \dots i'$ , two segments. The lower ones, included between this diagonal and  $\overline{AD}$ , have values respectively equivalent to 1, 2,  $\dots$  9  $\mu$ . If now from each point of intersection a line is drawn parallel to  $\overline{AD}$ , we would divide  $ABCD$  into 10 rectangles similar to  $AbcD$  in which the sides along  $\overline{AB}$  and  $\overline{DC}$ , such as  $\overline{Ab}$  and  $\overline{Dc}$  are each equivalent to 1  $\mu$ . The diagonals of these rectangle, such as  $\overline{Ac}$ , intercept on each of the perpendiculars  $a', b', \dots i'$ , two segments. In rectangle  $AbcD$ , the value of the lower one is, respectively equivalent to 0.1, 0.2,  $\dots$  0.9  $\mu$ . The homologous values for the rectangle immediately above are, 1.1, 1.2,  $\dots$  1.9  $\mu$ ; for the third rectangle, these values are 2.1, 2.2,  $\dots$  2.9  $\mu$ , etc.

In practice, only the diagonals of rectangles similar to  $AbcD$  are drawn by joining the numbered points of division of sides  $\overline{AB}$  and  $\overline{DC}$  as follows: Point O (=A) on  $\overline{AB}$  is joined to point 1 on  $\overline{DC}$ ; 1 on  $\overline{AB}$  to 2 on  $\overline{DC}$ , etc., as in part II of the figure.

To find the value in  $\mu$  of a dimension measured with dividers, one point of the dividers is run along  $\overline{AD}$  (part II) until the other point rests exactly on one of the diagonals. Assuming that, in an instance, one point of the dividers was at 0.3 as the other point rested exactly at point  $p$  of the diagonal 2, 3, the value of the dimension would be equivalent to 2.3  $\mu$ . For points between vertical lines, the values are estimated in centimicrons.

The length of  $\overline{AB}$  is obtained from the image of a stage micrometer photographed at the same magnification as the cells. The length of side  $\overline{AD}$  is arbitrary but usually made equal to 10 cm.

0.38 of the total width of the cell as obtained from the cell wall method. This ratio is much below the 2/3 ratio found by various investigators (see Knaysi, 1938), and even further below the ratios obtained from electron micrographs (Knaysi, 1942; Dubin and Sharp, 1944). This is probably due chiefly to the extreme youth of the cells used in the present experiments, and possibly somewhat to further shrinkage due to fixing by heat.

*Negative preparations.* A glance at figures 3 and 4 reveals that negative preparations, even in the absence of a thick slime layer, are unsuitable for the study of form and size of the bacterial cell. In negative preparations, the dimensions of the cell vary inversely with the thickness of the dye film. Measurement of 85 cells, in photomicrographs similar to those figures, shows cell widths ranging from 1.65  $\mu$  in the thin places to 0.68  $\mu$  in the thick places, with an average of 1.1  $\mu$ ; this average is, indeed, slightly less than those given by the two other methods but its value varies with the proportion of cells measured in thin and thick places. A glance at plate III of Dubin and Sharp's paper convinces one that these workers must have used mostly smears of sufficient thickness to

TABLE 1  
*Comparison between the values of cell width obtained by different methods*

METHOD	BACILLUS MYCOIDES		BACILLUS CEREUS	
	Range	Average	Range	Average
	$\mu$	$\mu$	$\mu$	$\mu$
Living cells in microcultures.....	1.13 to 1.20	1.17	1.68*	1.68
Cell wall method.....	1.07 to 1.25	1.16	1.60*	1.60
Negative-staining method.....	0.68 to 1.65	1.10		
Staining with Meyer's methylene blue.			0.5 to 0.75	0.61

\* Constant, or nearly so.

cause reduction in cell size. Consequently, the conclusion of Dubin and Sharp is correct for films exceeding a certain thickness, and our previous conclusion is correct for films below that critical value. Obviously, a method which depends on such an uncontrollable factor as the thickness of the dye film is not suitable for the study of size.

In view of the relation between thickness of film and cell dimensions, with which we have been familiar for many years, it becomes difficult to see how the explanation given by Dubin and Sharp of the relation between cell and dye film could account for the observed facts. Those authors examined minute glass cylinders in a dilute solution of Congo red. They state: "It is seen at once that dye tends to pile up or to be concentrated in the sulcus or angle formed where the cylinder lies on the slide. Under these conditions the negative image is bounded by dye lying beneath the actual cylinder and is therefore smaller than the cylinder." Although this concept is adequate to explain reduction in size, it cannot explain the experimental fact, clearly illustrated in figures 3 and 4, that when the dye film is below a certain thickness the cells appear larger than their true size.

Our own conception based on the existence of a meniscus which retracts upon drying (Knaysi, 1944) accounts for both reduction and increase in the apparent dimensions of the cell. Evidently, the glass cylinder and the bacterial cell do not have an identical behavior with respect to Congo red films.

#### SUMMARY

A comparative study of the cell width of *Bacillus mycoides* and *Bacillus cereus* shows that the value obtained depends on the treatment of the cells measured. Measurements of the living cells in the medium in which they are growing agree with those made on similar cells stained by a method showing the cell wall. In stained smears where the cell wall is not visible, the cells appear much smaller than they really are and represent the shrunken masses of the cytoplasm. In negative-staining smears, the cells may appear larger or smaller than their real size depending on the thickness of the dye film surrounding the cells. Consequently, negative-staining smears are not suitable for the study of cell dimensions.

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# THE INHERITANCE OF ENVIRONMENTALLY INDUCED CHARACTERS IN BACTERIA

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The problem of environmentally induced characters in bacteria is important both in theoretical and applied bacteriology. It is of special significance in chemotherapy, particularly as regards the development of sulfonamide and other drug-resistant forms. It is also of fundamental importance because of its bearing on microbial heredity.

A vast amount of literature is available indicating that bacteria and related organisms can become adapted to a wide range of environmental conditions. It is well established that by gradual progressive cultivation of bacteria in media containing increasing amounts of a toxic substance, a tolerant or resistant strain can in most cases be produced. On the other hand very little information is available on some of the fundamental questions which this phenomenon of "adaptation" raises. Can single cell cultures, therefore genetically pure, become adapted to different environments merely by being subjected to them? If so, what are the limits of their adaptive powers? Are the induced characters permanent and transmissible to subsequent generations, or are they of a temporary nature? What is the mechanism of adaptation? Is it a result of the selection to certain individuals occurring in the population, or do the toxic substances act as a direct stimulus upon the hereditary material of the cell, causing a new character or characters to appear?

## HISTORICAL

No references will be made to the literature dealing with induced characters other than that of resistance to drugs and toxic substances, since the experiments presented in this paper are limited to this particular phase. Much of the early work in this field consisted only of recording the adaptive phenomena. Danyz (1900) reported the development of a strain of *Bacillus anthracis* which would grow in solutions of arsenic five times as concentrated as that in which it would originally grow. Marks (1910) published a similar finding with *Salmonella paratyphi*.

Jollos (1913) working with paramecia, developed strains resistant to arsenic and noted that these resistant strains reverted to normal after varying lengths of time when cultivated in the absence of the drug. He suggested the term "Daurmodifikation" for these changes and stated that most bacterial variations are of this nature. Effront (1920) was able to develop a strain of beer yeast which possessed a tolerance to arsenic three times that of normal strains. He stated that production of resistant forms depends upon selection.

Richet, Bachrach, and Cardot (1922) studied in some detail the acquired toler-

ance of lactic acid bacteria for certain metallic poisons. They noted several things: (1) the adaptation was specific; (2) the organisms sometimes underwent other changes; (3) acquired characters once established maintained themselves for a large number of generations. Cardot and Langier (1923) reported work on the adaptation of lactic acid bacteria to potassium chloride. The modification produced was not specific since the resistant organisms also showed an increased resistance to other alkali and alkaline earth salts. The adaptation persisted through thousands of generations in a medium containing no potassium chloride. If the adapted and nonadapted strains were grown together in a medium without potassium chloride, the adapted strain was rapidly and completely eliminated by the other, thus showing selection in living competition. Jungeblut (1923) performed experiments on rendering *Vibrio cholerae*, *Eberthella typhosa*, and *Shigella dysenteriae* resistant to mercuric chloride; pneumococci to optochin; hemolytic streptococci to revanol and tryptaflavin; and staphylococci to methylene blue. In many cases the drug fastness was shown to be specific, but in a few cases "cross-fastness" was observed. The resistance to poisons was associated with an alteration in metabolism.

Feirer, Meader, and Leonard (1926) reported the development of drug-fast characters in strains of *Escherichia coli* and *Aerobacter aerogenes*. Using five germicides commonly used in the treatment of urinary infections, namely, mercurochrome, formaldehyde, acriflavine, silver nitrate, and hexrylresorcinol, they observed that a high degree of resistance was developed toward all these drugs. The resistant form was shown to be specific. Meader and Feirer (1926) in a later report state that the resistant strains lost their resistance after five days of cultivation in media containing no toxic substance. The development of strains of bacteria resistant to lysozyme action was reported by Fleming and Allison (1927). The resistance to lysozyme action was not specific in that bacteria made resistant to one tissue was equally resistant to all tissues and secretions. They also acquired resistance to the bactericidal power of the blood and to intracellular digestion by leucocytes.

Kafpus (1930) reported the development of strains of *Escherichia coli* resistant to malachite green. A thousandfold increase in resistance was observed. He further stated that the ability of an organism to develop in the presence of a poison did not affect its resistance to the killing action of the poison. He postulated that developmental resistance is obtained only if the bacteria are actively growing while under the effect of the disinfectant. Bacterial adaptation to acriflavin was studied by Burke, Ulrich, and Hendrie (1928). *Staphylococcus albus* showed increased tolerance for the antiseptic dye after 6 to 8 hours' exposure. This adaptation was temporary and disappeared when the organism was grown on dye-free agar. The increased tolerance was partially specific but not entirely so. Burke and Ulrich (1928) also worked with the adaptation of *Staphylococcus aureus* to gentian violet. They considered the changes purely an adaptation to a new environment and in no way associated with changes in the life cycle, dissociation of strains, selection of variant, or mutations.

In 1939 one of the first cases of "sulfonamide-fast" organisms was reported

by MacLean, Rogers, and Fleming. They observed that pneumococci in an infected animal treated with 2-sulphanihyl-amino-pyridine can readily establish a tolerance to the drug. Following this report many instances of sulfonamide resistance have been observed and recorded. MacLeod and Daddi (1939) produced a "sulfapyridine-fast" pneumococcus type I, by repeated transfers in broth containing increasing concentrations of the drug. The alteration was not associated with changes in morphology, virulence, or immunological characteristics. Doudoroff (1940) described the conditions under which adaptation of *Escherichia coli* to sodium chloride takes place most readily and discussed the various conditions affecting adaptive processes. Harris and Kahn (1940), working with sulfonamide-resistant organisms, reported that in some organisms the resistance induced by one drug is carried over to related drugs, in others it is not.

Lowell, Strauss, and Finland (1940) reported that strains of pneumococci initially susceptible to the bacteriostatic action of sulfapyridine, sulfathiazole, and sulfamethylthiazole were made resistant by growth in media containing increasing concentrations of these drugs. Strains accustomed in this manner to one of the drugs, not only acquired resistance of a high degree to the action of the homologous drug, but also became resistant to the other two chemicals to approximately the same extent. Individual strains varied in the ease with which resistance was acquired. Mulder (1940) reported that sulfapyridine-resistant strains developed *in vivo*. Strauss, Pringle, and Finland (1941a, 1941b) reported the development of sulfonamide-resistant strains of *Escherichia coli* and *Staphylococcus*. That sulfonamide-resistant strains of pneumococci were as sensitive to the action of penicillin as was the non-"sulfa-fast" parent strain was reported by Powell and Jamieson (1942). Rammelkamp (1942) has noted increased resistance of *Staphylococcus* to tyrothricin. Vivino and Spink (1942), McKee and Rake (1942), and Cooper and Keller (1942) have all reported the development of resistance to sulfonamide by various bacteria.

Schmidt, Sesler, and Dettwiler (1942) were able to develop one strain of type I and two strains of type III pneumococci highly resistant to sulfapyridine by serial passage through mice treated with less than curative doses of this drug. Resistance to the drug was retained by these strains for more than 200 passages through untreated mice. Sulfapyridine-resistant forms were also resistant to other sulfonamide derivatives.

The preceding review, although not complete, is sufficient to show that further work on adaptive phenomena is necessary to answer the questions raised in the introduction. It was with these questions in mind that the experiments reported here were undertaken.

#### MATERIALS AND METHODS

Single cell cultures of *Salmonella pullorum*, *Eberthella typhosa*, and *Salmonella schotmülleri* were cultivated in plain broth containing increasing concentrations of sodium chloride, mercuric chloride, and copper sulphate until a marked tolerance or resistance to a particular chemical was acquired. Following acquisition



of resistance to specific chemicals, single cell cultures were isolated, subcultures of which were transferred to media free of the chemical. These were then tested at approximately thirty-day intervals to determine whether the newly acquired character was transmitted to subsequent generations. Specificity of the resistance was determined by appropriate combinations of resistant strains and chemicals. Morphological and fermentative characters of the resistant strains were observed throughout the work.

Owing to difficulties encountered in the usual method of isolating single cells a modification was developed, which is similar to that used by a number of previous investigators. By means of a Chambers micromanipulator and micropipettes a small drop of bacterial suspension was placed on the underside of a sterile coverslip mounted above a moist chamber. Instead of attempting to remove drops containing single cells, the coverslips possessing such a drop were transferred to previously prepared moist chambers and incubated at 37 C for 24 hours. After incubation the descendants of the single bacterium could easily be obtained with an ordinary inoculating needle.

#### EXPERIMENTAL

*Adaptation to chemicals.* Single cell cultures of *Salmonella pullorum*, *Eberthella typhosa*, and *Salmonella schotmüller* were transferred to a series of culture tubes containing graded amounts of the various chemicals. The inoculated tubes were incubated at 37 C for 7 days. Tubes showing no turbidity at the end of this period were regarded as being completely inhibited. The highest concentration of chemical permitting growth was thus obtained. Following this preliminary work, transplants from the single cell cultures were grown in media to which was added progressively increasing amounts of the various chemicals. Table 1 shows the concentration of chemicals employed, the time required, and the ultimate degree of adaptation. It can be observed that in all cases a marked resistance to the chemicals employed was produced. Following adaptations to sodium chloride, the organisms were capable of growing in concentrations 2 to almost 3 times that which permitted growth of nonadapted cultures. In the case of copper sulfate a fivefold to sixfold increase in tolerance was noted, whereas the mercuric-chloride-resistant strains were able to develop in concentrations of mercuric chloride 6 to 12 times as great as that which permitted growth of the original cultures. Further training failed to yield strains of any greater resistance than those described above, although the final degree of resistance attained in all probability depends upon the particular species and chemicals employed.

To show clearly the differences between the resistant and nonresistant strains, plate counts were made of the two strains following inoculation of both strains into media containing the highest concentration of chemical permitting growth of the resistant strains. Table 2 gives the results obtained with the three strains of *Salmonella pullorum*. The other two species showed similar results and are therefore not included in table form. The data indicates clearly that the nonadapted strains are completely inhibited by concentrations of the chemical

which permits growth of resistant strains. The growth of the resistant strains, however, is somewhat retarded by the presence of the chemical, as can be readily seen by comparing the data of table 2 with that of table 3. In addition to showing the difference in rate of growth of resistant strains in the presence and absence of chemical, the data in table 3 also indicate that there is no difference in

TABLE 1

*Adaptation of Salmonella pullorum, Eberthella typhosa, and Salmonella schotmülleri to the presence of various chemicals*

ORGANISM	CHEMICAL	HIGHEST CONCENTRATION OF CHEMICAL PERMITTING GROWTH		PERIOD OF ADAPTATION
		Before adaptation	After adaptation	
<i>Salmonella pullorum</i>	NaCl	3%	8%	69
	CuSO <sub>4</sub>	1-4000	1-800	84
	HgCl <sub>2</sub>	1-300T	1-25T	101
<i>Eberthella typhosa</i>	NaCl	3%	6%	72
	CuSO <sub>4</sub>	1-4000	1-800	82
	HgCl <sub>2</sub>	1-300T	1-50T	82
<i>Salmonella schotmülleri</i>	NaCl	3%	8%	73
	CuSO <sub>4</sub>	1-4000	1-600	84
	HgCl <sub>2</sub>	1-300T	1-25T	72

TABLE 2

*Comparison of the growth of resistant and nonresistant strains of Salmonella pullorum*

HOURS AFTER INOCULATION	NUMBER OF BACTERIA PER MILLILITER (PLATE COUNT)					
	Broth containing 8% NaCl		Broth containing HgCl <sub>2</sub> 1-25,000		Broth containing CuSO <sub>4</sub> 1-800	
	Resistant	Nonresistant	Resistant	Nonresistant	Resistant	Nonresistant
0	231	2,500	386	896	360	410
12	720	137	1,267	506	450,000	400
24	97,740	270	33,000,000	291	39,000,000	300
36	21,000,000	270	166,000,000	78	172,000,000	210
48	102,000,000	80	103,000,000	0	102,000,000	160
60	23,000,000	40	48,000,000	0	46,000,000	160
72	18,300,000	23	40,000,000	0	30,000,000	120

growth rate of resistant and nonresistant strains in plain broth. Thus the acquisition of resistance to any of the chemicals employed in no way affected the ability of the organisms to grow in the absence of the chemical to which they had become acclimated. This fact is of considerable importance when one attempts to study the heritability of these characters and will be mentioned again in a later section.

*Specificity of resistant strains.* As was the case in the preceding section, only data for *Salmonella pullorum* will be presented since results obtained with the other two species are similar. To determine whether three different strains actually existed in regard to chemoresistance, the specificity of the resistant strains was studied. Table 4 presents the data obtained when the three resistant strains of *Salmonella pullorum* were introduced into media containing a chemical other than that to which they had been adapted. It is seen that a strain resistant to one chemical is no more resistant to the inhibiting action of the other chemicals employed than are the nontrained cultures. It can be assumed,

TABLE 3

*Comparison of resistant and nonresistant strains of Salmonella pullorum in plain broth*

HOURS AFTER INOCULATION	NONRESISTANT STRAIN	NaCl-RESISTANT STRAIN	HgCl <sub>2</sub> -RESISTANT STRAIN	CuSO <sub>4</sub> -RESISTANT STRAIN
0	390	310	503	430
12	580,000	556,000	623,000	1,400,000
24	123,000,000	106,000,000	100,000,000	148,000,000
36	202,000,000	205,000,000	212,000,000	259,000,000
48	125,000,000	100,000,000	146,000,000	70,000,000
60	78,000,000	66,000,000	87,000,000	56,000,000
72	39,000,000	23,000,000	34,000,000	33,000,000

TABLE 4

*Specificity of Salmonella pullorum resistant strains*

HOURS AFTER INOCULATION	NaCl-RESISTANT STRAIN			MERCURIC-CHLORIDE-RESISTANT STRAIN			COPPER-SULPHATE-RESISTANT		
	Broth + 8% NaCl	Broth + HgCl <sub>2</sub> (1-25T)	Broth + CuSO <sub>4</sub> (1-800)	Broth + 8% NaCl	Broth + HgCl <sub>2</sub> (1-25T)	Broth + CuSO <sub>4</sub> (1-800)	Broth + 8% NaCl	Broth + HgCl <sub>2</sub> (1-25T)	Broth + CuSO <sub>4</sub> (1-800)
0	1,200	1,400	1,100	1,700	2,100	1,700	1,900	2,600	1,800
12	31,000,000	700	900	1,000	42,000,000	1,500	1,100	1,800	26,000,000
24	149,000,000	400	990	450	189,000,000	1,000	960	750	174,000,000
36	108,000,000	100	560	500	146,000,000	950	800	450	110,000,000
48	96,000,000	0	910	350	101,000,000	1,000	1,100	150	89,000,000
60	51,000,000	0	740	400	69,000,000	860	950	0	42,000,000
72	33,000,000	0	810	250	41,000,000	720	600	0	23,000,000

therefore, that the changes recorded here are specific in nature and brought about in some manner by growing them in the presence of a particular chemical. It is probable, however, that the specificity of resistant strains is determined by the manner in which the chemical acts to inhibit growth. This view is supported by experimental evidence in the case of sulfonamide-fast organisms which are resistant to many sulfanilamide derivatives other than the one employed in the development of the resistant strain.

*Inheritance of chemoresistance.* The heritability of the newly acquired resistant characters was studied by isolating single cell cultures from the various

resistant strains, testing these cultures for resistance, transferring them to plain broth, and at regular intervals of time determining their ability to grow in the presence of the particular chemical to which they had been originally adapted. This procedure made certain that a pure resistant culture was obtained, avoiding the presence of dormant nonresistant organisms in cultures on which heredity studies were to be made.

Six single cell cultures were obtained from the salt-resistant strain and three from copper sulphate and mercuric chloride strains of *Salmonella pullorum*. Table 5 shows the results obtained with representative strains. After 18 months of propagation in plain broth, during which time they had been transferred 55 times, all single cell cultures of resistant strains retained their ability to grow in the presence of the chemical to which they had been adapted. Inasmuch as the 18-month sojourn in plain broth would represent thousands of generations, there can be no doubt as to the inheritance of the resistant characters.

TABLE 5

Comparison of growth of resistant strains after 18 months' propagation in plain broth and of nonresistant strains of *Salmonella pullorum*

HOURS AFTER INOCU- LATION	SODIUM CHLORIDE 8%		MERCURIC CHLORIDE 1-25T		COPPER SULPHATE 1-800	
	Non- resistant	Resistant	Non- resistant	Resistant	Non- resistant	Resistant
0	50,000	29,000	3,000	3,100	3,500	3,500
12	16,000	18,000	3,000	101,000	3,200	350,000
24	10,000	30,000,000	2,100	42,000,000	3,200	36,000,000
36	6,000	125,000,000	700	178,000,000	2,900	140,000,000
48	3,000	41,000,000	300	115,000,000	2,800	86,000,000
60	1,500	29,000,000	0	26,000,000	2,600	39,000,000
72	800	16,000,000	0	29,000,000	2,000	29,000,000

In the case of *Eberthella typhosa* and *Salmonella schotmülleri* plate counts were not made. It was simply noted whether growth occurred as judged by turbidity. Three single cell cultures of each of the resistant strains of these species were propoagated in plain broth for 9 months. During this time they had been transferred ninety times so that the actual number of generations involved is as large or larger than in the case of *Salmonella pullorum*. The results confirmed those obtained with the resistant strains of *Salmonella pullorum* in that all of the single cell resistant strains retained their resistance throughout the ninety transfers in plain broth.

A factor which may have contributed to the success of the resistant cultures in maintaining their resistance was the equal ability of the resistant strains to compete with the nonresistant strains in plain broth. In instances where induced characters are reported to have been lost rather soon when removed from the presence of the inducing agent, too frequently this factor has not been checked. If an organism possessing a newly acquired character, even though it were genetic in nature, is not able to compete on an equal basis with the original

organism, then even a low rate of reverse mutation would soon give rise to a population composed entirely of the original organisms due to selective action.

*Fermentative and morphological characters of the resistant strains.* Only the fermentative characters of *Salmonella pullorum* were studied in sufficient detail to warrant any conclusion. The parent strain of *Salmonella pullorum* coincided with the descriptions in Bergey's manual in respect to its physiological characters. The resistant strains did not in any case differ.

Nor was any marked change observed in cell morphology. In the case of the copper-sulphate-resistant strain of *Salmonella pullorum*, however, the cells were somewhat elongated.

*Mechanism of adaptation.* Of theoretical interest is the question of how an organism acquired resistance to growth-inhibiting action of various chemicals and drugs. Use of the single cell technique eliminated the possibility of resistant forms being present in the original cultures, so it must be assumed that the new forms arose from the original strain in direct response to the action of the chemical, or that they occurred spontaneously and that as a result of selective action

TABLE 6  
*Number of resistant forms obtained from normal populations*

ORGANISM	NUMBER OF COLONIES IN PLAIN AGAR	NUMBER OF COLONIES IN NaCl AGAR	NUMBER OF COLONIES IN HgCl <sub>2</sub> AGAR	NUMBER OF COLONIES IN CuSO <sub>4</sub> AGAR
<i>Salmonella pullorum</i> .....	800,000,000	15	21	48
<i>Eberthella typhosa</i> .....	440,000,000	61	32	30
<i>Salmonella schotmülleri</i> ..	480,000,000	120	16	29

produced a resistant strain. In seeking to test the latter theory the following experiment was devised:

Tests were made to ascertain the concentration of each of the chemicals in liquefiable solid media which prevented growth of the nonadapted strain, but which allowed the resistant strain to grow. This medium was then heavily seeded with the nonadapted strain. If the original population contained a small number of resistant individuals, they would develop in this medium to the exclusion of nonresistant forms. By appropriate dilutions and controls it was found that normal populations of bacteria did contain a few individuals which were able to grow when plated in agar containing the various chemicals. Table 6 presents the results obtained in this phase of the study. The colonies which developed were transferred to broth containing the highest concentrations of chemical employed, where they grew without difficulty. Thus it was established that in the population of a given species of bacteria there are individual cells which possess resistance to a particular chemical, and which by appropriate technique can be isolated and a resistant culture obtained without a long period of training.

A possibility which should not be overlooked is that perhaps the resistant forms were not present as such in the original population, but that they were

capable of responding to the action of the chemical. In other words, the hereditary mechanism of a few cells might have been susceptible to alteration by the chemical used. How this point can be settled is not apparent, since testing for chemoresistance necessitates placing the organism in contact with the chemical. The fact still remains, however, that certain cells differ in their reaction to the presence of chemical agents. It therefore is probable that adaptation of bacteria to various environmental conditions consists of selection of such individuals.

#### DISCUSSION

In view of the controversy as to the nature of bacterial variation it seems appropriate to consider the results obtained with reference to various theories. It is well known that variability of living organisms in general can be divided into two distinct components: the nonheritable modifications and the heritable variations. Almost all cases of well-analyzed hereditary differences between individuals have proved to depend upon combinations of Mendelian "unit characters," and it is known that all segregating unit differences are due, in respect to their origins, to mutations. It can thus be said that mutations and their combinations constitute the basis of almost all analyzable hereditary differences.

A mutation may be defined as a change in heritable characters, leading from one more or less stable condition to another. They are known to occur spontaneously, i.e., without application of any special mutation-inducing factors in all species of plants and animals thus far analyzed in this respect. There is therefore good reason to believe that spontaneous mutation is a general characteristic of all living things. Furthermore, extensive observations of the mutation process in plants and animals have shown that all known types of morphological and physiological character changes can be produced by mutations. Mutant characters vary from ones so slight that they can be detected only by means of special methods up to changes so profound that they may produce death or serious pathological symptoms.

Differences in mutation rates of different genes are far greater than can be accounted for by chance, indicating that certain genes are more susceptible to change than others. This fact may account for the ease with which bacterial cultures develop resistance to certain drugs as compared to other drugs. The same is true of reverse mutations; certain mutant genes have never been observed to revert, whereas, on the other hand, some mutate with equal frequency in either direction. It is also known that mutation rates may differ in different strains of the same species.

The bacterial variations reported in this paper are best explained by assuming that they are mutations. The specificity and retention of their chemoresistance support this theory. It seems unlikely that they represent phases in the life cycle, in view of their permanency. Furthermore, the large number of drugs to which a species has been reported resistant, to say nothing of other types of variation, would make the number of phases in the life cycle so numerous that this theory becomes untenable.

## SUMMARY AND CONCLUSIONS

Single cell cultures of *Salmonella pullorum*, *Eberthella typhosa*, and *Salmonella schotmülleri* were able to adapt themselves to concentrations of sodium chloride, mercuric chloride, and copper sulphate considerably higher than those required to inhibit growth of nonadapted strains.

The resistant strains were specific in their ability to overcome the growth-inhibiting properties of each chemical.

Single cell cultures of resistant strains retained their resistance for long periods of time and through numerous transfers. Their permanency and specificity suggest a change in hereditary constitution; thus are probably mutations.

Experiments showed that populations of the bacteria studied contain a few individuals which possess the ability to withstand the action of the chemicals employed without previous contact with the chemical.

In view of the hereditary nature of the changes and their specificity it seems logical to classify them as mutations. Furthermore, the fact that these resistant forms are found in small numbers in normal populations lends further support to this idea. It may be concluded that the adaptation of strains of bacteria to certain adverse environmental conditions probably consists of selecting from the population mutants which possess this resistant character, thus giving rise to a strain composed entirely of resistant individuals.

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# PENICILLIN

## IV. A DEVICE FOR PLACING CYLINDERS ON ASSAY PLATES

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When large numbers of assays for antibiotics, such as penicillin, are to be conducted by the cylinder plate method (Abraham *et al.*, 1941; Schmidt and Moyer, 1944), the time consumed in performing certain of the numerous routine operations, such as placing the cylinders in the proper position on the test plates, comprises an appreciable portion of the working day. The authors have constructed and used a simple device which greatly speeds up this operation and places cylinders in exactly reproducible patterns on the agar plates; this device is described here in the hope that it will be found useful by other workers engaged in such work. The apparatus, which is shown in figure 1, operates in the following manner:

A petri dish containing agar which has been inoculated with the test organism, such as *Staphylococcus aureus* or *Bacillus subtilis*, is placed in a closely fitting depression on a vertically movable wooden platform (plate holder) which is pivoted at the end distant from the handle. The petri dish is thus brought into a position about one inch below the five glass delivery tubes. The plate holder is lifted by means of the handle until it is stopped by the adjustable locked nuts which are visible at the left of the petri dish in figures 1 and 2. As it rises, the plate holder, operating through a metal lever arm, causes the cylinder-release mechanism to discharge one cylinder within each of five troughs and, simultaneously, to bear against and to retain the adjacent cylinder in each trough. The five released cylinders each slide into the five glass delivery tubes, which are arranged to convey the respective cylinders to the five predetermined loci on the agar plate. The ends of the glass delivery tubes are approximately  $\frac{1}{4}$  inch above the agar when the cylinders are delivered. The cylinders used in this device may be of glass (8 mm O.D. x 10 mm) or aluminum ( $\frac{5}{16}$ " O.D. x 0.394"). The wall thickness of the glass or aluminum tubing is about 1 mm.

When the plate holder containing the now loaded petri dish is lowered, the pressure of the spring locks against the bottom cylinder in the respective troughs is released, and the columns of cylinders descend a distance equivalent to the length of one cylinder; the five cylinders which are to be placed on the next dish are then brought into the proper position for release when the plate holder is again raised.

The mechanism by which the columns of cylinders are retained during the release of the five cylinders is shown in figures 2, 3, and 4. Small brass bolts,

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with flat heads  $\frac{1}{4}$  inch in diameter, are fitted with coil springs and held to the assembly plate by means of brass nuts. The pressure exerted against the retained cylinders is that exerted by the springs.



FIG. 1. CYLINDER-PLACING DEVICE

Figure 3 shows the short metal tips at the lower end of the release mechanism that hold the five cylinders in the release chamber until the release mechanism is tripped. Figure 4 shows the apparatus at the moment of release of the cylinders, which may be seen descending through the glass tubes. It will be noted that the cylinders which are to be released during the next cycle are still retained by the spring-stop mechanism; they will fall to the next position (retention by the metal tips) when the plate holder is lowered.

The apparatus is separable at a position just above the release mechanism, and an empty magazine may be removed and replaced with a loaded, sterile unit

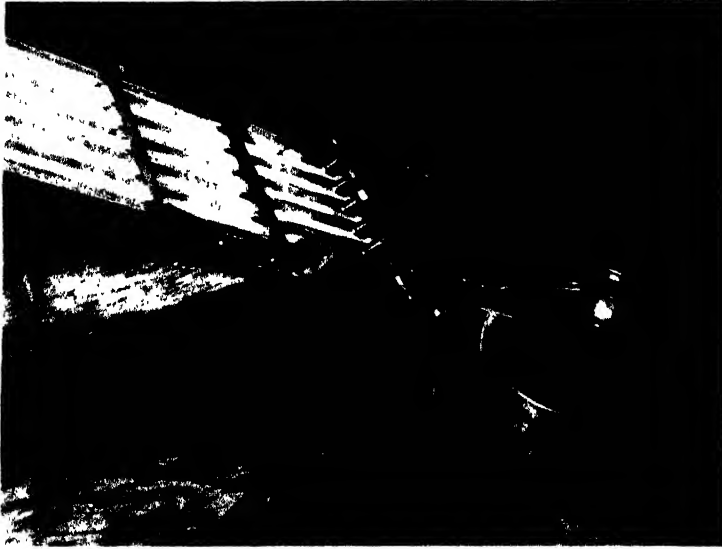


FIG. 2. (Top.) VIEW OF CYLINDER-RELEASE MECHANISM, SHOWING SPRING RETAINERS  
 FIG. 3. (Lower left.) VIEW OF CYLINDER-RELEASE MECHANISM, SHOWING RETAINING TIPS  
 FIG. 4. (Lower right.) VIEW OF DEVICE IN OPERATION, SHOWING CYLINDERS DESCENDING  
 GLASS TUBES AND BEING PLACED ON AGAR SURFACE

(figure 5). Until the magazine is mounted and ready for operation, the cylinders contained in it are held in the troughs by an endgate, which is shown in the closed position in figure 6 and in the open position in figure 7.

The magazine is attached to the apparatus by means of two small clips or prongs which fit snugly over the two sides of the block holding the cylinder-

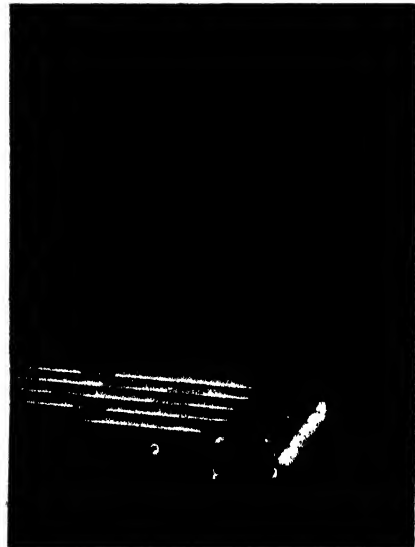
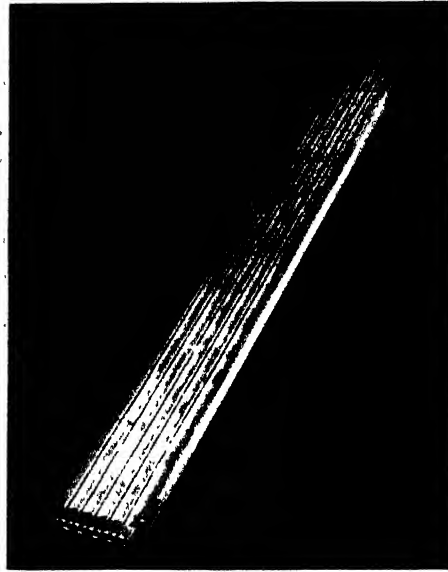


FIG. 5. (Top.) LOADED MAGAZINE, ENDGATE CLOSED

FIG. 6. (Lower right.) END OF MAGAZINE, SHOWING ENDGATE IN CLOSED POSITION

FIG. 7. (Lower left.) END OF MAGAZINE, SHOWING ENDGATE IN OPEN POSITION

release mechanism. These prongs are shown at the right end of the magazine in figures 6 and 7. The upper end of the magazine is prevented from moving laterally by two wooden strips which are attached to the sides of the vertical

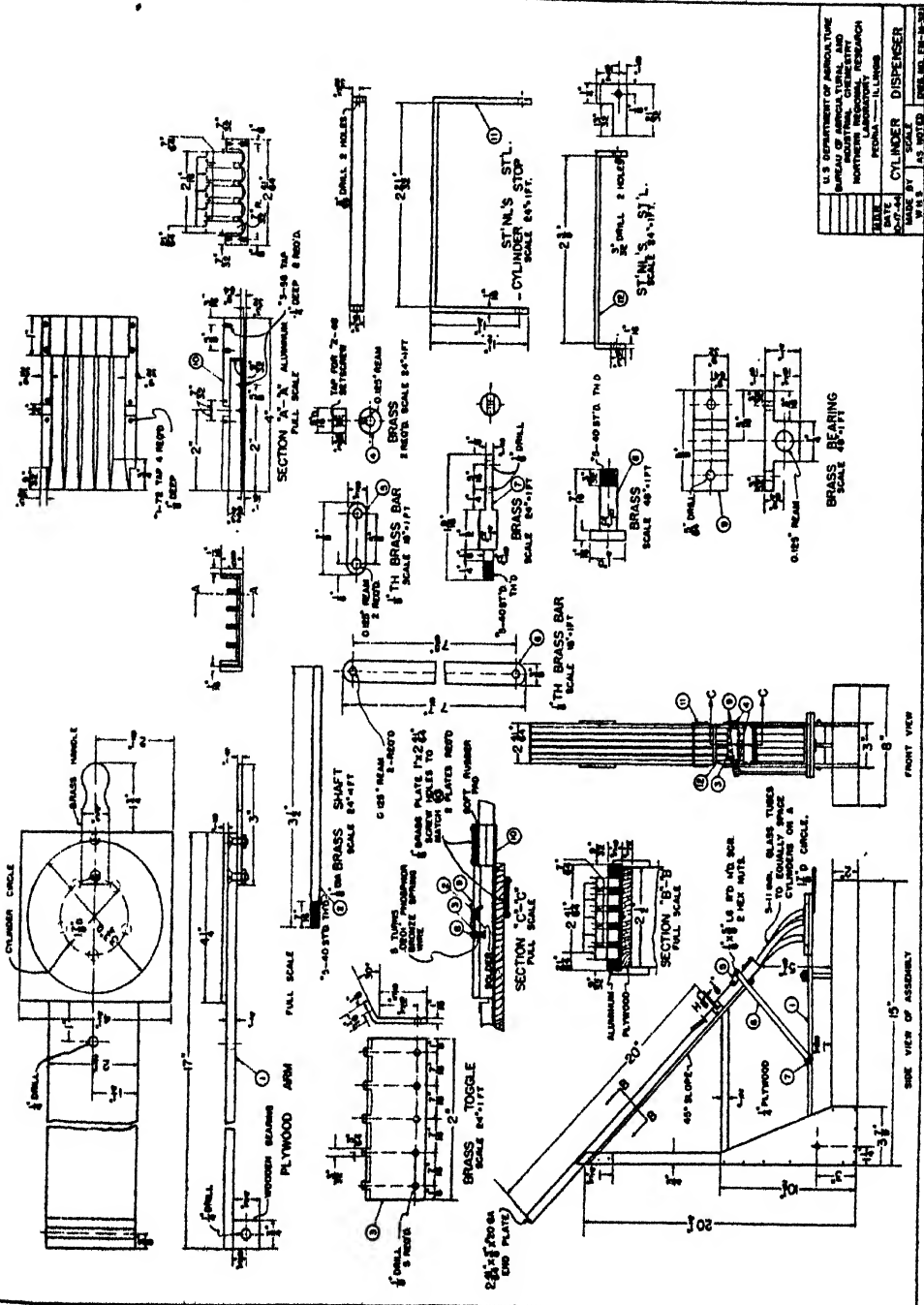


Fig. 8. Details and Assembly of Cylinder-Placing Device

supporting member of the framework. Before use, the loaded magazines are sterilized for approximately one hour at 170 C. Under the working conditions of this laboratory, we have found it unnecessary to sterilize the release mechanism and glass tubes.

In figure 8 are shown details of each part and views of the assembled device. The main framework and the plate holder of the unit constructed and used by the authors are made of wood. The cylinder-delivery tubes are pyrex glass, 9 mm inside diameter, and are held firmly in place by a rubber gasket beneath the rectangular brass plate which is adjusted by four screws (figure 3). The magazines and the trough unit conducting the cylinders to the glass delivery tubes are fabricated from aluminum blocks, the troughs being machined out. The trough unit and attached cylinder-release mechanism are secured to the wooden framework by a clamp, which is visible in figure 2.

The cylinder-release mechanism, comprising the screws, springs, and retaining tips, is made of brass, as is also the lever arm operating between the plate holder and the cylinder-release mechanism. Variation in the movement of the cylinder-release mechanism may be obtained by changing the point of attachment of the lever arm to the plate holder.

It has been observed that one person, using this device, can place cylinders on about 12 plates per minute. Three or four skilled operators are required to handle plates at the same rate if the cylinders are individually placed by means of tweezers. This apparatus has a further advantage in that the cylinders fall upon the agar with approximately the same force and at the proper angle, thus reducing the number of leaky and otherwise improperly placed cylinders.

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# A PARTIALLY DEFINED MEDIUM FOR CULTIVATION OF PNEUMOCOCCUS

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A chemically defined medium capable of supporting the growth of pneumococcus would be of value in studies on the metabolism and sulfonamide resistance of strains of this organism and would simplify the isolation of metabolic products. Before a medium may be considered to satisfy the nutritional requirements of a microorganism it must meet two qualifications—it must permit a heavy growth of the organism starting with a reasonably small inoculum, and it must permit unlimited subculture of the organism.

Rane and Subbarow (1940) have developed a gelatine hydrolyzate medium which supported growth of four strains of pneumococcus, but failed to permit growth of a fifth strain, and no report of subculture on this medium was made. Cohen, Halbert, and Perkins (1942) reported poor growth and "failure of growth in successive transplants" on the medium of Rane and Subbarow. In our own experience none of ten strains tried would grow on subculture in this medium. Rane and Subbarow demonstrated the essentiality of pantothenic acid, nicotinic acid, and choline for the strains they tested. A mixture of known amino acids could replace the gelatine hydrolyzate as a medium for three of the strains but not for the fourth. Asparagine was included in the amino acid mixture but was not added to the gelatine hydrolyzate. Glutamic acid was added to both media. This addition is of interest in view of the observation of Fildes and Gladstone (1939) that in the absence of glutamine, growth of a strain of pneumococcus was greatly delayed or failed completely, whereas with glutamine growth occurred overnight. They noted that glutamine could be replaced by a high concentration of glutamic acid. Bohonos and Subbarow (1943) recently demonstrated that pneumococcus requires biotin for growth, a fact predicted by Landy *et al.* (1942) from observations on a single strain. Bohonos and Subbarow used an acid hydrolyzate of casein supplemented with, among other substances, glutamine, choline, and asparagine. They noted difficulty in the subculture of certain strains on this medium.

Badger (1944) also found the medium of Rane and Subbarow to be inadequate for several strains of pneumococcus, and in investigating the nutritional requirements of a strain of pneumococcus type III developed a medium similar to that of Bohonos and Subbarow and similar in most respects to media developed for the cultivation of group A hemolytic streptococci by Bernheimer *et al.* (1942) and by Woolley (1941).

All of these media for pneumococcus contain a reducing agent such as ascorbic acid (Badger, 1944), or thioglycollic acid (Bernheimer *et al.*, 1942), in accordance with the findings of Dubos (1929) that a lowered redox potential is essential for



the initiation of growth of the pneumococcus. None of these media for pneumococcus contains added  $\text{CO}_2$  despite the findings of Kempner and Schlayer (1942) that the lag phase in the growth of pneumococci is an inverse function of the  $\text{CO}_2$  concentration in the medium, being infinite at concentrations below 0.1 per cent. The observation of Badger (1944) that the "ageing of the basal medium (for two days in the refrigerator) shortened the lag period considerably" is undoubtedly explained by the solution of  $\text{CO}_2$  from the air during this "ageing."

#### EXPERIMENTAL

**Medium.** Since the medium of Rane and Subbarow (1940) proved to be unsuitable for the cultivation of our strains of pneumococcus, we turned to the medium of Bernheimer *et al.* (1942), designed for the growth of the C203S strain of hemolytic streptococcus group A. This medium was prepared using an acid hydrolyzate of Harris' vitamin-free casein as a base in place of the acid digest of technical casein used for streptococcus. This medium failed to support growth of pneumococcus, but on addition of all the usual growth factors growth was obtained, and by a process of elimination the essential factors missing from the C203S medium were found to be choline and asparagine. The essentiality of choline has been demonstrated both by Badger and by Rane and Subbarow, but the essentiality of asparagine for pneumococci has not been demonstrated, although it was included in the media both of Badger and of Bohonos and Subbarow. Apparently not all strains of pneumococcus require asparagine as well as glutamine. The complete medium is described in detail in table 1 and will hereafter be referred to as the pneumococcus medium.

**Inoculum.** Eight-hour cultures of pneumococci in beef heart infusion broth were centrifuged and resuspended in sterile saline. Each 10-ml tube of the pneumococcus medium was inoculated with one loopful of washed organisms, and at the same time a tube of neopeptone broth received an identical inoculum as a control on the viability of the seeding culture. The inoculum for serial subcultures in the pneumococcus medium was one loopful of unwashed culture. Broth controls were set up simultaneously using an inoculum of the same size.

**Test of Medium.** Ten strains of pneumococci were tested on the casein hydrolyzate medium of Bernheimer *et al.* (1942), made up as above, but lacking choline and asparagine. Growth was absent or scanty on first inoculation in the medium, and no strain grew on second transfer. However, on the addition of as little as 0.1 ml of meat infusion neopeptone broth to 10 ml of the casein hydrolyzate medium fair growth was obtained, and with 1 ml, excellent growth. This would indicate that failure of growth in the medium of Bernheimer *et al.* is due to lack of some nutrient rather than to the presence of a toxic component which might render it an unsuitable pabulum for pneumococcus. Accordingly, a "shotgun" mixture of amino acids and growth factors consisting of glutathione, methionine, norleucine, hydroxy proline, beta alanine, threonine, pimelic acid, inositol, folic acid, sodium oleate, asparagine, choline, and *p*-aminobenzoic acid was added to the Bernheimer medium, and fairly good growth of pneumococcus occurred.

TABLE 1

*The pneumococcus medium***Basal Medium—for one liter of medium**

Acid hydrolyzate of casein.....	200	ml of 10% solution
<i>l</i> -Cystine.....	150	mg
<i>l</i> -Tryptophane.....	20	mg
KCl.....	3	gm
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O.....	7.5	gm
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.5	gm
Distilled water to make.....	900	ml

Adjust pH to 7.5, heat to boiling, filter, and tube in 9 ml amounts or appropriate multiple.  
Autoclave.

**Solution I—vitamin mixture for 12.5 liters**

Biotin.....	0.015	mg
Nicotinic acid .....	15.0	mg
Pyridoxine.....	15.0	mg
Calcium pantothenate.....	60.0	mg
Thiamine.....	15.0	mg
Riboflavin .....	7.0	mg
Adenine sulfate.....	150.0	mg
Uracil.....	150.0	mg

Dissolve in 100 ml of distilled water and sterilize by filtration. Store in refrigerator.

**Solution II—salt mixture for 50 liters**

FeSO <sub>4</sub> ·7H <sub>2</sub> O .....	50	mg
CuSO <sub>4</sub> ·5H <sub>2</sub> O .....	50	mg
ZnSO <sub>4</sub> ·7H <sub>2</sub> O.....	50	mg
MnCl <sub>2</sub> ·4H <sub>2</sub> O.....	20	mg
HCl concentrated.....	1	ml

Dissolve in 100 ml of distilled water and sterilize by boiling.

**Addition mixture per liter of medium**

Vitamin mixture (solution I).....	8.0	ml
Salt mixture (solution II).....	2.0	ml
Glucose (20% solution).....	10.0	ml
Glutamine.....	200	mg
Asparagine.....	100	mg
Choline.....	10	mg
CaCl <sub>2</sub> ·2H <sub>2</sub> O.....	10	mg
Distilled water to make.....	50	ml

Sterilize by filtration and store in refrigerator. Add 0.5 ml to each 9 ml of basal medium.  
This addition mixture should not be kept longer than a few weeks as the glutamine is unstable. Solutions I and II appear to keep indefinitely.

**Bicarbonate—thioglycollate mixture**

Thioglycollic acid.....	10%
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Add 1 ml of thioglycollic acid to 9 ml of sterile distilled water, mix well and heat in boiling water bath for 10 minutes.

Bicarbonate. Weigh 200 mg samples of sodium bicarbonate into test tubes and autoclave. Add 10 ml of sterile distilled water to a test tube containing bicarbonate and dissolve the latter. Then add 0.2 ml of 10% thioglycollic acid and mix well immediately. Add 0.5 ml of the mixture to each 9.5 ml of medium. This bicarbonate-thioglycollate mixture is unstable and must be made up and added to the medium just prior to inoculation.

The nonessential growth factors were excluded by a process of elimination. The crucial experiment of the series is given in detail as follows: The medium was that of Bernheimer, to which had been added all the previously mentioned additional growth factors except choline, asparagine, and PABA. The latter were added both singly and together. To control tubes, 0.1 ml of neopeptone broth was added instead of the three substances under test. All tubes were set up in duplicate. Visible growth was estimated roughly after 18 hours of incubation, and final pH was determined as an additional indication of growth. The test organism was a rough variant of pneumococcus type I. The inoculum was one loopful in 10 ml of medium, equivalent to approximately  $10^{-8}$  ml of whole culture. The results of these tests are given in table 2.

TABLE 2  
*Test for essentiality of choline, asparagine, and PABA*

NUMBER	ADDITION	VISIBLE GROWTH	FINAL pH
1	None	0	7.6
1a	None	0	7.55
2	Infusion peptone broth, 0.1 ml	+++	6.8
2a	Infusion peptone broth, 0.1 ml	++	6.9
3	Choline, one loopful of 0.1% solution	+	7.2
3a	Choline, one loopful of 0.1% solution	++	7.1
4	PABA, one loopful of 0.1% solution	0	7.5
4a	PABA, one loopful of 0.1% solution	0	7.6
5	Asparagine, 2 mg	±	7.4
5a	Asparagine, 2 mg	±	7.3
6	Choline, PABA, and asparagine	+++	6.8
6a	Choline, PABA, and asparagine	++++	6.6

As shown in table 2, both asparagine and choline possess growth-promoting qualities for pneumococcus. PABA is inert in this respect. When all three substances were present growth was excellent.

Further experiments eliminated PABA, the amino acids, and growth factors from consideration, indicating that with an inoculum of  $10^{-8}$  ml or larger, choline and asparagine are the only essential growth requirements for this strain of pneumococcus which are not present in the medium of Bernheimer *et al.*

In order to rule out the possibility that the growth-promoting effect of asparagine might be due to some contaminating substance, the asparagine (cp) was twice recrystallized from water and on comparative titration proved slightly more effective in promoting growth than the original material. The pneumococcus medium was used except that asparagine was added separately and the glucose concentration doubled to permit heavier growth and thus accentuate

any difference between cp and recrystallized asparagine. The data are presented in table 3.

The results of the experiment outlined in table 3 indicate that for this strain of pneumococcus at least asparagine is an essential growth factor in a glutamine-containing medium. The strain used by Badger apparently required neither glutamine nor asparagine, but either substance shortened the lag phase and accelerated growth.

The essentiality of glutamine was demonstrated for three freshly isolated strains of pneumococci, types III, V, and IX. The medium used was the complete medium described in table 1 but with glutamine omitted. The organisms were centrifuged, washed once in saline, and resuspended in saline to the original volume. After inoculation with 0.1 ml of washed culture into 10 ml of glutamine-free medium, types V and IX grew well in 16 hours whereas type III grew well

TABLE 3

*Comparison of cp asparagine and twice recrystallized asparagine as growth factors for IR strain of pneumococcus\**

TUBE	ADDITION	VISIBLE GROWTH	FINAL pH
1	None	+	6.8
2	None	+	6.8
3	0.1 ml neopeptone broth	++++	5.2
4	0.1 ml neopeptone broth	++	6.5
5	1 mg asparagine cp	++++	5.1
6	1 mg asparagine (2× recrystallized)	++++	5.0
7	0.1 mg asparagine cp	++++	5.25
8	0.1 mg asparagine (2× recrystallized)	++++	5.50
9	0.01 mg asparagine cp	++	6.3
10	0.01 mg asparagine (2× recrystallized)	++	6.8
11	0.001 mg asparagine cp	+	6.9
12	0.001 mg asparagine (2× recrystallized)	+	7.0

\* Tubes were seeded with one loopful of a broth culture of IR pneumococci which had been centrifuged and resuspended in saline to one-fifth the original volume.

only after 48 hours. None of the three strains grew in subculture on the glutamine-free medium. In other experiments the lag period before initiation of growth was found to be a function of the size of the inoculum and the amount of glutamine in the medium, becoming infinite with small inocula and reduced amounts of glutamine. The limiting quantities in each case are a characteristic of the strain used. It is evident, however, from the work of Badger that one strain of pneumococcus at least can be carried for 20 transfers in a glutamine-free medium.

*Subculture in medium.* The pneumococcus medium, using an acid hydrolyzate of vitamin-free casein as a base, was tubed in 10 ml amounts. The culture was a mouse-virulent strain of pneumococcus type I (SVI). Using an inoculum of 0.1 ml, the culture was readily carried in duplicate through 9 transfers in the medium, full growth taking place after incubating for about 5 hours. With an

inoculum of one loopful ( $10^{-8}$  ml), both tubes in the duplicate series did not always show growth within 24 hours, but by the inoculation of two tubes from the tube in which growth had occurred after 24 hours, the culture was carried through 6 transfers in the medium.

For many purposes a vitamin-free base is not necessary. Accordingly, the pneumococcus medium was prepared using a commercially available acid hydrolyzate of casein, "casamino acids" (Difco). On this medium two freshly isolated strains of pneumococci as well as an old laboratory strain were readily carried for 10 transfers, using 0.1 ml inoculum for each transfer in 10 ml of medium. In addition 23 other strains of pneumococci have been grown for one or more transfers in the medium. No attempt was made to carry these strains for many transfers. In fact, we have found only one strain of pneumococcus, a markedly degraded rough variant of type III (M3R), which did not grow well on this medium. Failure of this strain to grow indicates that a medium satisfactory for all strains of pneumococci is not yet available.

*Use of medium in investigations of sulfonamide resistance of pneumococci.* As MacLeod (1940) has pointed out, it is most important in studying sulfonamide susceptibility of strains of pneumococci to employ a medium free of sulfonamide inhibitors. Meat infusion and the various peptones are heavily contaminated with inhibitors, and as a consequence routine bacteriological media are unsuited for this purpose. The pneumococcus medium, prepared with vitamin-free casein hydrolyzate base, is inhibitor-free. Certain lots of casamino acids (Difco) were found to be very low in inhibitor, but other lots contained so much that they were unusable for studies of sulfonamide susceptibility. It might be well to note at this point that different lots of casamino acids vary greatly also in the content of lactic acid and fermentable carbohydrates. If these substances are to be avoided it is necessary to use specially purified casein for preparing the acid hydrolyzate.

Tillett, Cambier, and Harris (1943) used the pneumococcus medium, prepared with casamino acids low in inhibitor, in testing sulfonamide-fast strains of pneumococcus for their ability to synthesize sulfonamide inhibitors. They refer to it as the medium of Bernheimer *et al.* (1942). It should be noted that choline and asparagine were also present in the medium which they used.

When testing for sulfonamide susceptibility or resistance by determining the ability of an organism to grow in various concentrations of sulfonamides, it is important that small inocula be used. With the pneumococcus medium, growth is uncertain and the lag phase long when inocula smaller than  $10^{-8}$  ml of whole culture are used. However, the addition of fresh rabbit serum, shown by MacLeod (1940) to be sulfonamide-inhibitor-free, makes it possible to initiate growth with inocula as small as  $10^{-8}$  ml of culture, containing 1 to 5 organisms, as determined by pour plates. The pneumococcus medium containing casamino acids and enriched with 0.25 ml of rabbit serum per 10 ml was used to compare the growth of a sulfonamide-susceptible strain, SVI, with a sulfonamide-resistant strain, P86, in the presence of various concentrations of sulfathiazole. The results of these tests are shown in table 4.

As shown in table 4, the sulfonamide-susceptible strain, SVI, grows poorly in  $M/16,000$  and not at all in  $M/8000$  sulfathiazole. The resistant strain, P86, however, grows well in  $M/1000$  sulfathiazole. One sample of each culture was washed before dilution to remove sulfonamide inhibitors derived from the meat infusion peptone broth. Washed and unwashed cultures behaved identically in the test, as might be expected, since the inoculum was  $2 \times 10^{-6}$  ml, too small a volume to contain significant amounts of inhibitor.

Different lots of medium were tested directly for the presence of sulfonamide inhibitors by the method of MacLeod (1940). In this method, the effect of added substances suspected of containing inhibitors of sulfonamide bacteriostasis of *Escherichia coli* grown in a defined medium is determined. The pneumococcus medium made with one lot of casamino acids contained inhibitor equivalent to a  $10^{-6}$  M PABA solution, a negligible amount. As is noted above, some lots

TABLE 4

*Growth of sulfonamide-susceptible and sulfonamide-resistant strains of pneumococci in the presence of various concentrations of sulfathiazole*

STRAIN OF PNEUMOCOCCI	CONCENTRATION OF SULFATHIAZOLE							
	$M/1,000$	$M/2,000$	$M/4,000$	$M/8,000$	$M/16,000$	$M/32,000$	$M/64,000$	None
SVI....	—	—	—	—	++	++++	++++	++++
SVI washed culture....	—	—	—	—	+	++++	++++	++++
P86....	++++	++++	++++	++++	++++	++++	++++	++++
P86 washed culture....	++++	++++	++++	++++	++++	++++	++++	++++

Medium: The pneumococcus medium enriched with 2.5% rabbit serum.

Culture: An 18-hour broth culture diluted in the pneumococcus medium. Washed culture is the same culture centrifuged and resuspended to original volume in the pneumococcus medium and then diluted.

Inoculum:  $2 \times 10^{-6}$  ml per 10 ml of medium; with SVI, 300 organisms, and with P86, 600 organisms by plate count.

Incubation: 22 hours at 37 C.

of casamino acids contain so much inhibitor as to be unusable for this purpose. Vitamin-free casein hydrolyzates contain no detectable inhibitor.

*Use of medium for growth of hemolytic streptococcus.* The medium of Bernheimer *et al.* (1942) was designed for growing the C203S strain of hemolytic streptococcus and appears to be a complete medium for this strain. In our hands, some freshly isolated group A strains grew well, whereas other strains failed to grow even in the medium supplemented with choline and asparagine. However, when the complete pneumococcus medium was supplemented with 2.5 per cent rabbit serum, all strains of group A hemolytic streptococci which were tested grew well from small inocula. Such an enriched medium has been used by Wilson (1944) for investigations on the sulfonamide resistance of group A strains freshly isolated from individuals treated with sulfonamides prophylactically or therapeutically.

*Use of the medium for massive growth of pneumococcus.* Bernheimer *et al.* (1942) report the massive growth of several strains of group A hemolytic streptococci on the medium devised by them. In an experiment based on this procedure, massive growth of the SVI strain of pneumococcus type I was obtained using the medium of table 1 prepared with casamino acid base. To one liter of this medium and to one liter of meat infusion neopeptone broth as a control were added 5 mg of phenol red as an indicator. The flasks were inoculated with 5 ml of an 18-hour culture of pneumococci and incubated at 37 C. As soon as acid production became evident, as judged by a marked change in the indicator

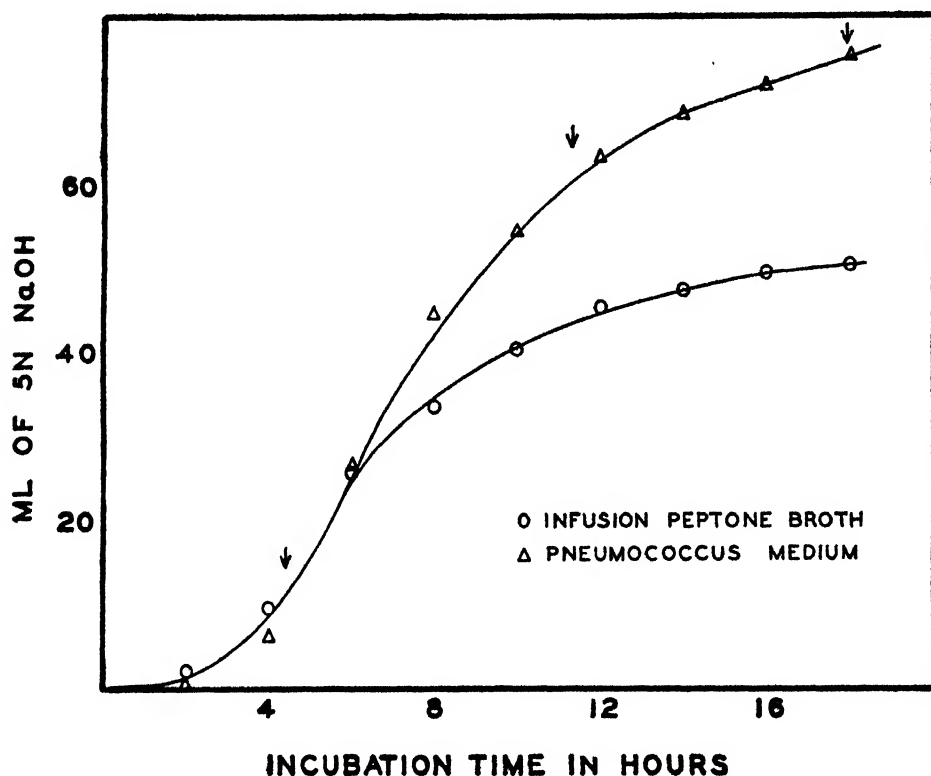


FIG. 1. Amount of sodium hydroxide required to maintain at pH 7.5 cultures of pneumococci growing in infusion peptone broth and in the pneumococcus medium.

color, additional glucose to a final concentration of 5 per cent was added to each flask. The pH was maintained at about 7.5 by frequent additions of 5N NaOH. In a period of 17 hours (figure 1) 75 ml of 5N NaOH were required to maintain the pH of the casamino acid medium, whereas only 50 ml of 5N NaOH were required for the broth control (figure 1). It is evident that the casamino acid medium permits a heavier growth of pneumococci than does the broth. According to Bernheimer *et al.*, the C203S strain of hemolytic streptococcus required about 50 ml of 5N NaOH per liter of medium when grown under similar conditions. Smears were made at the times indicated in the figure to determine the

degree of autolysis of the organisms in the two media. In general there was less autolysis in the casamino acid medium than in broth. If intact organisms are desired, smears should be stained at intervals during the course of growth so that the organisms can be harvested before autolysis has progressed too far. The pneumococcus medium, being free of nondialyzable substances, facilitates the isolation of such metabolic products as the capsular polysaccharides and bacterial enzymes.

#### SUMMARY

A medium is described which permits growth of most strains of pneumococcus in repeated subculture. The medium is completely dialyzable and can be prepared free of sulfonamide inhibitors. The utility of this medium for obtaining massive growth of pneumococcus and for studies on the sulfonamide resistance of pneumococcus and hemolytic streptococcus has been demonstrated.

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# PENICILLIN

## VI. EFFECT OF DISSOCIATION PHASES OF *BACILLUS SUBTILIS* ON PENICILLIN ASSAY

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In the bioassay of penicillin, inhibition of the growth of a penicillin-sensitive microorganism is usually taken as the criterion of potency, the comparison being made against a standard of known activity. During the past year, it has become apparent that the majority of penicillin samples are mixtures of at least two and sometimes three distinct chemical entities, each of which has its own spectrum of biological activity against a variety of microorganisms. These three entities, which are known as penicillins F, G, and X in this country, and I, II, and III, respectively, in England, often occur in widely differing proportions in various samples.

As one of the possible consequences of this situation, a given sample of penicillin will exhibit different apparent potencies depending on the test organism used and on the composition of the standard penicillin with which comparison is being made. It is with the intention of assisting in the clarification of this problem that this note is being submitted.

During recent months, all assays in this laboratory have been made against a standard consisting of pure sodium penicillin G. This compound has recently been chosen as the international standard, of which 0.6  $\mu$ g corresponds to one international unit. With this salt as a standard, the pure sodium salts of penicillins F and X have been assayed against *Staphylococcus aureus* NRRL B-313 (FDA strain 209P), *S. aureus* NRRL B-314 (Heatley strain), and *Bacillus subtilis* NRRL B-558 (both smooth and rough phases) by means of the cylinder-plate assay method. Typical results are shown in table 1. The data can be used to calculate the ratios of the activities of the various penicillins against the organisms in question:

	PENICILLIN F	PENICILLIN X
Activity vs. <i>B. subtilis</i> (smooth).....	0.65	0.95
Activity vs. <i>S. aureus</i> (313)		
Activity vs. <i>B. subtilis</i> (rough).....	0.65	1.42-2.00
Activity vs. <i>S. aureus</i> (313)		

In view of these different ratios, when a given sample of penicillin is assayed against these three organisms, it is possible to draw tentative conclusions as

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TABLE 1

ORGANISM	PENICILLIN G	PENICILLIN F	PENICILLIN X
	<i>units per mg</i>		
NRRL B-313.....	1667*	1490	845
NRRL B-314.....	1667*	1440	935
NRRL B-558 (smooth phase) ..	1667*	970	800
NRRL B-558 (rough phase) ..	1667*	970	1200-1700

\* By definition.

to the proportions of the various penicillins present. Such conclusions involve the assumption that there are present no other substances possessing antibacterial activity. If the rough phase of *B. subtilis* is used, each day's inoculum must be standardized against a known pure penicillin X, since some variation in the sensitivity of this dissociated strain has been encountered, presumably because of variation in the degree of dissociation. In presenting these data, no claim is made that this method affords a precise means of analysis; it is believed, however, that results thus obtained are in most cases indicative of composition.

These observations indicate clearly that assay values obtained when *B. subtilis* is used as the sole test organism are valid only when the unknown and standard penicillins are comprised of the same proportions of the various chemical entities which are known to give the "penicillin" response. Moreover, the necessity of giving due consideration to the phase of the test organism is apparent. It is especially interesting that when an organism changes from the smooth to the rough phase, the response to one penicillin increases by almost 100 per cent, although the response to other penicillins remains constant. Aside from their bearing on the assay of penicillin, these findings suggest that an understanding of strain and phase susceptibility may be of fundamental significance in the clinical application of antibiotic compounds such as penicillin.

# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## NEW YORK CITY BRANCH

### THIRTY-FIRST MEETING

GEORGE WASHINGTON HOTEL, NEW YORK CITY,  
DECEMBER 28, 1944

UNSTABLE STRAINS OF *ESCHERICHIA COLI-MUTABILE*. *Stephen Zamenhof*, New York City.

The author has isolated two unstable strains of *Escherichia coli-mutabile* which "mutate" to each other with a high frequency but without forming papillae. One of the strains is a slow lactose fermenter and forms long chains; the other behaves like typical *Escherichia coli*. Taking into consideration the effect of chain formation, the "mutation" rate has been estimated at 1 per 1,000 to 5,000 individual cell divisions (Rec. Gen. Soc. Am., 13, 41).

Further study reveals also the nonhereditary variations such as mucus, capsule, and alkali formation, all induced by alkali produced by the other cells; these variations are interwoven in a rather complicated pattern with the above-mentioned hereditary changes ("mutations").

A SPECIFIC LYTIC SUBSTANCE IN *ESCHERICHIA COLI*. *Stephen Zamenhof*, New York City.

The filtrates of cultures of a smooth strain of *Escherichia coli* (no. 4657, Am. Type Culture Collection) contain a substance which lyses *specifically* the cells of another rough strain of *E. coli* (no. 4656), but is entirely harmless against any of several other tested strains of *E. coli* (both rough and smooth), or against staphylococci and gram-positive bacilli. Filtrates of the rough strain (no. 4656), or of the several other rough and smooth strains of *E. coli*, are harmless against smooth strain no. 4657 and against several other smooth and rough strains of *E. coli*.

This *specific lytic substance* (SLS) is not a phage. Neither is it a specific polysaccharide. It is destroyed entirely by heating for 15 minutes at 81 C, is 99.9 per cent de-

stroyed by 71 C for 15 minutes, and practically not affected by 61 C for 1 hour. It is not destroyed by standing for 1 month at 25 C.

The action of the substance is not bacteriostatic but bacteriolytic. The yield of SLS in 1 ml of 24-hour-old broth cultures of strain no. 4657 is potent enough to kill in 24 hours an inoculum of 15,000 cells of strain no. 4656; inocula 50 times smaller are killed within 10 minutes. However, quantities of SLS 100 times larger are entirely harmless to any other rough or smooth strains of *E. coli*, staphylococci, or gram-positive bacilli. Such an absolute specificity of lytic action is not known in the bacterial products found hitherto, except for a case described by Gratia (1925).

BACTERIOLOGICAL STUDY OF CANNED EVAPORATED MILK IMPLICATED IN AN OUTBREAK OF GASTROENTERITIS. *A. G. Osler, E. Dillon, and L. Buchbinder*, Bureau of Laboratories, New York City Department of Health.

From 43 cans of evaporated milk presumably concerned in an outbreak of acute food poisoning the following bacterial counts were obtained: greater than 100 per ml—24 (12 cans showed no abnormality); greater than 1,000 per ml—14 (5 normal in appearance); greater than 1 million per ml—9 (2 normal). Abnormal-appearing cans showed leakage, swelling, or rusting, with accompanying changes in appearance and odor of the milk.

No *Shigella*, *Salmonella*, *Clostridium*, or *Staphylococcus aureus* bacteria were isolated.

*Streptococcus faecalis* was present in greatest number and in most of the cans, occurring in pure culture in some. A study of 49 strains of this organism showed the following characteristics: gram-positive diplococ-

cus or short-chained streptococcus producing alpha type hemolysis on sheep blood agar; milk acidified and coagulated; good growth at 10 C and 45 C; growth in presence of 0.1 per cent methylene blue and in 6.5 per cent NaCl; gelatin not liquefied; not completely destroyed by exposure to 60 C for 30 minutes. Acid without gas is produced from mannitol, dextrin, arabinose, maltose, mannose, amygdalin, trehalose, fructose, galactose, lactose, salicin, sucrose, and glycerol. In glucose broth pH is lowered to about 4.6. Delayed fermentation of rhamnose may occur. No change in inulin, inositol, dulcitol, adonitol, xylose, raffinose, erythritol, or sorbitol.

The imputed relationship of this streptococcus to a food poisoning outbreak is of added interest because of the occasional presence of *S. faecalis* in pasteurized milk. The question is raised of its possible etiologic rôle in food poisoning of "unknown" origin.

#### THE DESTRUCTION OF THERMOPHILIC BACTERIA BY ULTRAVIOLET RAYS. *Louis Lang*, National Sugar Refining Company.

Work at the National Canners Laboratory showed the need for sugar free from thermophiles, particularly for canning nonacid vegetables, that is, peas, corn, sweet potatoes, etc. Sugar Refiners developed such sugar, meeting the exacting specifications of National Canners by ultra-filtration of the sugar liquors. With the advent of the ultra violet tube, producing a maximum radiation in the range of 2,537 Å units, these lamps were tested. Experiments have proved that when sugar is placed in single layers, the thermophiles are destroyed by an instantaneous exposure. Since thermophiles may be in the crystal as well as on the under side of the crystal, it is evident that this very short exposure penetrates the crystal.

Apparatus has been designed for the application of this lamp to the sugar problem. In one case, a continuous belt with lamps at 4-inch centers is used. In another installation a 10-foot stainless steel slide with lamp mounted above is functioning perfectly. In still another case, a vibrating surface conveyor transports the sugar from inlet to outlet. Above the conveyor are lamps at 4-inch centers. These conveyors are satisfac-

torily producing approximately one ton of sugar per hour, and this sugar meets the National Canners' specifications.

#### QUANTITATIVE ESTIMATES OF THE BACTERIAL FLORA OF ENCLOSED PLACES IN NEW YORK CITY. *Leon Buchbinder, Mathilde Solowey, and Morris Solotorovsky*, Bureau of Laboratories, New York City Department of Health.

Approximately 5,000 samples of air in schools, subway cars, theaters, streets, and a park were taken during a period of one year to determine quantitatively the total bacterial flora.

It was found that schools averaged more organisms per cubic foot of air than any of the other types of location studied. The latter can be arranged in decreasing order of magnitude as follows: subway cars, non-air-conditioned theaters, streets, air-conditioned theaters, and a park. Differences between the several types of locations are largely a matter of variation in dust and bacteria associated with dust, occupancy, and ventilation.

Schools showed greater variation than did any of the other types of locations. These differences, in part at least, could be ascribed to variability in their original construction. Variations in the counts were found in each of the schools according to whether a room was a classroom or an assembly room, occupied or vacant.

When the subway car findings were divided arbitrarily into two groups, one high and one low according to the number of persons present, they showed differences which were fairly consistent throughout the year. Bacterial counts in subway cars also varied according to the hour of the day. These differences also seemed to be a measure of occupancy, both present and recent.

A correlation in schools between the total bacterial counts and alpha hemolytic streptococcus counts is indicated, and it is suggested that the total bacterial count may be used as an indirect index of nasopharyngeal contamination in such places.

#### RECENT APPLICATIONS OF BACTERIOLOGICAL CONTROL IN THE FOOD INDUSTRIES. *Daniel Melnick and Bernard L. Oser*, Food Research Laboratories.

During the past decade interest in bacteriological control in the food industry has been stimulated by new food, drug, and cosmetic legislation, new processing methods and equipment, growth of the concept of quality control, the introduction of microbiological assays for vitamins and amino acids, and finally the special needs created by the war. The recent expansion of plant inspection activities of the Food and Drug Administration and the opposing views regarding a tolerance for filth were discussed. Among the processing operations receiving increased attention are sterilization and pasteurization by flash heating and more recently by the application of high-frequency currents; ultraviolet irradiation for antirachitic and bactericidal purposes; homogenization; low-temperature freezing and storage; and improved methods and equipment. The advantages of specificity and of relative simplicity of microbiological assays were discussed, and the need for study of the optimum conditions for preparing extracts of foods for assay was emphasized. These advances in food technology and control, as well as the improved assay techniques, introduce problems which demand close coordination of bacteriology with other sciences, particularly biochemistry.

THE CULTIVATION OF *STREPTOCOCCUS MITIS* ("STREPTOCOCCUS VIRIDANS"). George H. Chapman, Clinical Research Laboratory.

*Streptococcus mitis* grows more slowly and less luxuriantly in artificial culture media than do the other common streptococci of man. It is inhibited by sodium chloride in concentrations above 0.2 per cent and requires the phosphate ion as an essential metabolite. Ability to utilize nitrogenous cleavage products varies with the strain, best results being obtained with a mixture of tryptose, proteose peptone, and proteose no. 3 peptone. Azo N is utilized. The specific toxic antigen required for optimum stimulation of antibodies is produced much more plentifully in the presence of brain and heart infusion. The addition of "vitamin B complex" does not accelerate growth when the requirements listed above are met. Some strains grow hardly at all in otherwise adequate mixtures, and the missing factor is being sought.

LABORATORY PROCEDURE IN THE DIAGNOSIS OF GONOCOCCAL INFECTIONS. Alfred Cohn, Bureau of Laboratories, New York City Department of Health.

The applicability and limitations of laboratory methods in the diagnosis of gonococcal infections were discussed with reference both to the clinical course of the disease and also to the laboratory facilities at present available. It is the chronic disease which presents a complicated picture. Cultures are much more reliable than smears for diagnosis, but a negative diagnosis must be substantiated by repeated cultures, as the possibility of a transitory latent infection must be considered.

The sensitivity of the culture media employed is not so great as would be desired. The basis for most gonococcus media is proteose peptone no. 3 Difco agar; modifications of this medium and various enrichments added to the basic media have improved its sensitivity for the growth of the gonococcus. The methods used for transportation of pus specimens were mentioned. The value of the gonococcus complement fixation was discussed.

The entire subject of laboratory methods in the diagnosis of gonococcal infections is constantly under investigation, and improvements in our methods will undoubtedly be arrived at, especially because venereal disease research is urgently prompted by the present war conditions.

NON-ACID-FAST FORMS OF *MYCOBACTERIUM TUBERCULOSIS* AS SHOWN BY A NEW STAINING TECHNIC. Eleanor Alexander-Jackson, Department of Public Health, Cornell University Medical College.

Non-acid-fast forms of *Mycobacterium tuberculosis* have been variously interpreted as degenerative forms, filterable elements, artifacts, and young forms which have not yet developed acid fastness.

Kahn's demonstration that granule forms of *M. tuberculosis* could sprout or elongate into young rods which matured into classical acid-fast tubercle bacilli was confirmed by the author. Unsatisfactory staining methods led to the author's improved counter stain (Science, 99, 307). After staining by the Ziehl-Neelson technique, and decolorization with acid alcohol, this in-

volves flooding with Loeffler's methylene blue, addition of a few drops of normal NaOH, washing, and brief application of sodium hydrosulfite as a bleach. The blue color is selectively removed from all of the smear except the non-acid-fast forms of *M. tuberculosis*. Other non-acid-fast organisms and tissue cells are then stained green.

The triple stain revealed a hitherto undemonstrated zooglycal form of the tubercle bacillus, which has been studied by means of single-cell and electron microscopic methods (in press).

The exact rôle of non-acid-fast forms of *M. tuberculosis* in tuberculous infections remains to be determined, but may be significant. The triple stain should be of value in their study and in examination of clinical material showing few or no typical acid-fast rods.

**THE RELATIONSHIP OF SULFONAMIDES TO PENICILLIN ACTION.** *Gladys L. Hobby, Charles Pfizer and Co., and M. H. Dawson, College of Physicians and Surgeons.*

*In vitro* experiments indicate that factors which determine the effect of sulfadiazine on penicillin action include the organisms involved, their number, environmental conditions, susceptibility to both penicillin and sulfadiazine, and the concentration of each bacteriostatic agent.

In a concentration of penicillin producing little or no bacteriostasis, sulfadiazine increases bacteriostasis only if the organism is sulfadiazine-sensitive and present in small numbers. This bacteriostatic effect is predominantly due to sulfadiazine. In concentrations of penicillin producing a definite bactericidal effect during early incubation, but not complete sterilization, sulfadiazine is bacteriostatic only if the number of organisms present at the end of the sulfadiazine lag period is low and they are sensitive to this agent. An excess of penicillin acts rapidly during the first few hours of incubation, and complete sterilization may result before the end of the sulfadiazine lag period. If sterilization is incomplete at the end of this lag period, sulfadiazine will decrease the rate of growth, provided the organism is sensitive to sulfadiazine and present in small numbers. Because penicillin acts predominantly at the time of cell division, a de-

creased rate of multiplication due to sulfadiazine tends to decrease the rate at which penicillin acts.

Determination of the enhancement of penicillin action by sulfadiazine in human infections must await the result of clinical trials.

**INTERFERENCE BETWEEN SIMIAN AND MURINE STRAINS OF POLIOMYELITIS VIRUS.** *Claus W. Jungeblut, College of Physicians and Surgeons.*

**BACTERIOLOGICAL STUDIES IN ENDOCARDITIS.** *Ward J. MacNeal and Anne Blevins, New York Post-Graduate Medical School and Hospital.*

Inflammations of the endocardium may be nonbacterial or bacterial. Expanding knowledge of anti-infectious agents has increased the hope of successful therapy of infections of the blood stream and of the endocardium, and has stimulated study of the bacteria concerned and their susceptibility to anti-infectious agents.

Blood cultures should be repeated at least 3 successive days in both liquid and solid media. Previous antibacterial therapy may require the addition of inactivating agents to the medium. Several subcultures are compared and tested for behavior toward anti-infectious agents.

From 36 patients with endocarditis the following organisms were isolated during the past year: *Streptococcus salivarius*, 13; *Streptococcus equinus*, 7; *Streptococcus faecalis*, 6; *Streptococcus bovis*, 3; viridans (unspecified), 2; micrococcus (unspecified), 2; *Staphylococcus aureus*, 1; *Actinomyces septicus* (N.S.), 1; gram-negative rod (unidentified), 1. This series is not considered representative.

Streptococci of the *salivarius*, *equinus*, and *bovis* groups were generally susceptible to penicillin and resistant to bacteriophages. Those in the *faecalis* group were generally resistant to penicillin and susceptible to bacteriophages. The various bacteria exhibited marked differences in susceptibility to neoarsphenamine and sodium bismuth thioglycollate.

The possible development of drug fastness during therapy presents considerable difficulty and is not yet fully elucidated.

**A WARTIME STUDY OF CIVILIAN WOUNDS.**

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This was a brief summary of certain observations made under the Contaminated Wound Project of the Sub-committee on Surgical Infections of the Division of Medical Sciences of the National Research Council. The work was done under a contract recommended by the Committee on Medical Research between the O. S. R. D. and Columbia University.

Complete clinical and laboratory observations were made in nine eastern, southern, and midwestern hospitals on 2,493 cases of compound fractures, lacerations, and burns, emphasizing the bacteriology of such wounds and testing experimentally under controlled conditions the local and systemic use of certain sulfonamide drugs.

A central laboratory was established to recheck all cultures difficult to identify, particularly anaerobes, to type streptococci, and to study experimentally the efficacy of commercial gaseous gangrene serums.

Many species of microorganisms, both aerobic and anaerobic, find their way into wounds. Many of these are nonpathogenic, but the pathogenic anaerobes are much more common in debrided tissues than the relatively small number of anaerobic infections under civil conditions indicates. There were only 8 cases of gaseous gangrene and 2 of tetanus, but there were many infections attributable to streptococci and staphylococci.

The observations made in the clinical studies were unfavorable to the local use of sulfonamides in civil wounds.

Seven commercial gaseous gangrene serums were shown to possess the indicated antibodies; all had highly prophylactic values in experimental guinea pig infections.

**CAN CHEMOTHERAPY BE EXTENDED TO INCLUDE INTRACELLULAR PARASITES?**

*Stuart Mudd*, University of Pennsylvania.

This paper will appear in the *Journal of Bacteriology* as the Presidential Address.





# THE BIOCHEMICAL AND SEROLOGICAL RELATIONSHIPS OF THE ORGANISMS OF THE GENUS *PROTEUS*

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There has been marked confusion and disagreement in definitions and limitations of the genus *Proteus* since it was established by Hauser in 1885. Our review of the literature will be limited to those reports which contributed or should have contributed much to the modern concept of this genus. Hauser (1885) noted the swarming characteristics of these organisms and named two species *Proteus vulgaris* and *Proteus mirabilis*, the former liquefying gelatin rapidly and the latter more slowly. Theobald Smith (1893), who was the first to study the action of *P. vulgaris* in glucose, lactose, and sucrose, emphasized two important characteristics of this organism. First, its powers of gas production were "peculiar" since a much smaller gas volume was formed in glucose in 24 hours by *P. vulgaris* than by "*B. coli-communis*." His tables show *P. vulgaris* produced a gas volume in 24 hours of only 4 per cent, whereas several strains of "*B. coli*" isolated from different sources produced from 28 to 46 per cent volumes in the same time. Moreover, he showed that certain species now classed as *Salmonella* produced gas volumes comparable to *Escherichia coli*, whereas species of *Aerobacter* yielded even greater volumes of gas. Second, he noted that the fermentative powers of "*B. coli-communis*" differed from those of *P. vulgaris* in that the former produced acid and gas in glucose, lactose, and sucrose, whereas the latter failed to attack lactose. In 1894 Smith, finding that old *Proteus* cultures showed a diminished or complete loss of power to liquefy gelatin, cited the fermentation of glucose and sucrose and the absence of lactose fermentation as a particular characteristic of *Proteus* organisms.

Most unfortunately, subsequent investigators, almost without exception, established as a cardinal character of the genus *Proteus* the fermentative reaction recommended by Smith (1894) without, however, reference to the small gas volume produced by such organisms (Smith, 1893). In the present investigation of 454 *Proteus* cultures studied in detail and about 200 additional cultures differentiated routinely in this and neighboring laboratories, none has produced more than from a bubble to approximately 15 per cent gas in 48 hours in any fermentable carbohydrate. Shell vials, 7 mm in inside diameter by 28 mm long, were used in fermentation tubes.

Wenner and Rettger (1919) studied 73 *Proteus* strains with a fairly wide range of biochemical reactions. Their cultures swarmed and liquefied gelatin. They pointed out, however, that the liquefying property of a culture might be entirely lost. The 73 cultures fermented glucose, sucrose, and occasionally maltose, but not lactose. All cultures produced hydrogen sulphide, and 69 digested casein. No strain produced any pigment on agar. This work, which

was taken as a point of departure in the American system of classification, divided *Proteus* into two distinct groups. Strains attacking glucose, sucrose, and maltose readily were *P. vulgaris*, and those strains attacking glucose rapidly, sucrose slowly, and not fermenting maltose were called *P. mirabilis*.

In 1927 Moltke made a comprehensive study of 194 *Proteus* cultures. In addition to the characteristics established by Wenner and Rettger (1919), Moltke demonstrated that all his cultures attacked urea rapidly and many fermented salicin. It is curious that considerable controversy arose about the work of Wenner and Rettger (1919) and Moltke (1927) despite the fact that where the same tests were used, their results (except for indole production) agreed very closely.<sup>1</sup> Taylor (1928) studied 53 *Proteus* cultures isolated principally from urinary and wound infections and feces. All cultures swarmed, liquefied gelatin, and peptonized milk. Lactose and mannitol were not attacked by any culture. In glucose "acid and a small quantity of gas were produced in 24 hours and no change occurred subsequently. This reaction was uniform

<sup>1</sup> Wenner and Rettger (1919) stated: "Of 73 strains studied, 25 showed an immediate and 48 a delayed reaction (in sucrose). In correlating these results with the action in other carbohydrates, it soon became apparent that the strains which fermented sucrose readily also fermented maltose, while those which showed a delayed action on sucrose did not attack maltose." The authors conclude their paper with the statement, "For the species fermenting this sugar (maltose) the name *Proteus vulgaris* is suggested and for the species failing to attack it the name *Proteus mirabilis*."

In view of the foregoing quotations it is difficult to understand two statements made by Moltke (1927) in referring to the work of Wenner and Rettger (1919): "Twenty-five (of the 73) strains fermented maltose at once, none later on. It is not stated whether these 25 strains are the same as those fermenting saccharose at once." And, "In the maltose fermentation Wenner and Rettger see a possibility of sub-grouping, but they do not attempt such."

Thirty-seven of Moltke's 194 strains fermented maltose and 157 did not. The maltose-positive strains fermented sucrose rapidly and the maltose-negative ones slowly. In Moltke's summary under "Fermentations by *Bact. proteus* . . .," it is stated: "Their action on maltose divides the *Proteus* bacilli into two sharply distinguished fermentation groups. . . . The maltose fermenting strains ferment saccharose at once; the non-maltose fermenting strains ferment saccharose but slowly." Nevertheless in Bergey *et al.* (5th ed., 1939) and elsewhere is found, "However, Moltke . . . and others do not recognize this description (Wenner and Rettger's) and state that most *Proteus vulgaris* strains are maltose negative." This, we believe, arose from a misinterpretation of Moltke's writing. The term "*Bact. proteus vulgaris*" appears in the title of Moltke's paper and seldom thereafter. The term "*Proteus*" *mirabilis* appears only once and then in a direct reference to Hauser's work. "*Bact. proteus*" and "*Proteus* bacilli" are used almost exclusively throughout the paper, giving us the impression that he considered "*Bact. vulgaris*" or the "*Proteus* bacilli" as a group rather than a species. To assume, therefore, that the species *P. vulgaris* does not ordinarily ferment maltose because 157 of Moltke's 194 strains of *Proteus* bacilli did not ferment maltose does not seem to be what this author meant to imply.

One reason is apparent to explain Moltke's failure to support Wenner and Rettger's division of the genus into two groups according to the fermentation of sucrose and maltose. Wenner and Rettger (1919) found no correlation between fermentation reactions and indole production. Moltke, on the other hand, found almost perfect correlation between the two reactions. All but one of Moltke's 37 maltose-positive strains (*P. vulgaris*) were indole-positive whereas the 157 maltose-negative strains (*P. mirabilis*) were indole-negative. The present investigation is in entire agreement with Moltke with respect to indole production.

in every strain." Sucrose was fermented by the 53 cultures. In a majority of cases this reaction was delayed. Three cultures fermented maltose and these were the only indole-positive strains. Of Taylor's 53 cultures 3, or 5.7 per cent, were *P. vulgaris*, and 50, or 94.3 per cent, were *P. mirabilis*. The low ratio of *P. vulgaris* isolates to *P. mirabilis* isolates is a characteristic distribution of these two species.

Rauss (1936) examined 48 cultures of Morgan's bacillus no. 1 and concluded that this organism belonged to the genus *Proteus*. He found that such cultures swarmed under suitable conditions, formed indole, and fermented glucose consistently and sucrose occasionally. Both the American (Bergey *et al.*, 5th ed.) and British (Topley and Wilson, 1936) systems classify this organism as *Proteus morganii*.

In 1904 Rettger isolated from chickens during a choleralike epidemic a previously undescribed organism. Some years later this culture together with others was sent to Hadley who was working on the paratyphoid and related organisms (Rettger, personal communication). Hadley *et al.* (1918) named the unidentified culture "*Bacterium Rettgeri*." Since that time this organism has undergone the following taxonomic changes: *Bacillus* (St. John-Brooks and Rhodes, 1923), *Eberthella* (Bergey, 1st ed.), *Shigella* (Bergey, 5th ed.), and finally *Proteus* (Rustigian and Stuart, 1943a). Edwards (1942, personal communication) found it to be motile. Rustigian and Stuart (1943a) confirmed the motility of this species; showed that it hydrolyzed urea strongly and rapidly, and that some strains swarmed under suitable conditions; and proposed that the organism be classified as *Proteus rettgeri*. Even in this laboratory, however, independently isolated strains of this organism were called "anaerogenic paracolon type 33111" (Stuart *et al.*, 1943a), then "*P. entericus*" (Rustigian and Stuart, 1943b) before our strains finally were identified as *P. rettgeri*.

Prior to the division of the genus *Proteus* into two species by Wenner and Rettger (1919) there was little agreement concerning the antigenic relationships among *Proteus* organisms. Some workers found that only the homologous strain agglutinated in an antiserum, whereas others found an antiserum agglutinated a number of heterologous strains to high titers. Wenner and Rettger showed that of 73 cultures of *P. vulgaris* and *P. mirabilis* only 19 failed to agglutinate in one or another of a total of 7 antisera prepared against the two species. All except 1 antiserum agglutinated heterologous cultures.

Moltke (1927) studied the H and O antigens of his cultures. By direct agglutination tests 3 main groups were established, whereas adsorption tests divided the 3 groups into a number of subgroups. The nonswarming strains lacked H antigens and exhibited a variety of O antigens. Rauss (1936) found that the H antigens of *P. morganii* had group specific and the O antigens type specific tendencies. He established 7 different H and 17 different O antigens in his 48 strains. An H antigen common to *P. morganii* and *P. vulgaris* was discovered. The first cultures of *P. rettgeri* studied showed marked serological homogeneity (Stuart, Wheeler, *et al.*, 1943, anaerogenic paracolon type 33111), but as more strains were studied antigenic heterogeneity was encountered.

Strains of this species were found which failed to agglutinate in an antiserum, but upon adsorption completely removed all agglutinins. Similar findings for other species of *Proteus* have been reported by Taylor (1928) and by Welch and Poole (1934).

### *Sources of Cultures*

A total of 454 cultures of *P. vulgaris*, *P. mirabilis*, *P. morganii*, and *P. rettgeri* was studied. Of 69 *P. vulgaris* strains, 29 were isolated from feces, 6 from urine, 2 from osteomyelitis cases, and 32 were received from stock cultures of many different laboratories. Of 205 *P. mirabilis* strains, 118 were isolated from feces, 32 from urine, 34 from such sources as ureter, blood, lung, kidney, bladder, chest fluid, ileum, peritoneum, intestinal mucosa, wounds, and burns, and 21 were received from stock culture collections. One hundred and two *P. morganii* cultures were studied; 65 from feces, 7 from urine, 17 from such sources as stomach, kidney, scalp, and wounds, and 13 were obtained from culture collections. Of 78 *P. rettgeri* strains, 77 were isolated from fecal specimens and one from blood.

During the 4-year period of this investigation 190 cultures of *P. vulgaris* and *P. mirabilis* were isolated or received by us shortly after isolation from human sources. Of the 190 cultures, 14.3 per cent were *P. vulgaris* and 85.7 per cent were *P. mirabilis*. The marked difference in the relative incidence of the two species agrees with Levine (1942) and with Dammin and Belling (1942) who found that 85 to 90 per cent of the *Proteus* bacteria isolated from humans were *P. mirabilis*.

To determine the frequency of *Proteus* in normal feces, 110 specimens were examined. Specimens were plated directly on eosin methylene blue (EMB) agar and on *Salmonella* and *Shigella* ((SS) agar, and also inoculated into either selenite or tetrathionate enrichment broth. After 24 to 36 hours' incubation the enrichment broths were plated on EMB and SS agar. On direct plating 10 per cent of the specimens yielded *Proteus*, 8.2 per cent *P. mirabilis* and 1.8 per cent *P. morganii*. From preliminary enrichment broth 36.3 per cent of the specimens were positive; 23.6 per cent *P. mirabilis*, 10 per cent *P. morganii*, and 2.7 per cent *P. vulgaris*. Hynes (1942) examined 353 fecal specimens from typhoid and paratyphoid patients. With direct plating on desoxycholate agar 10 per cent of the specimens yielded *Proteus*, whereas with tetrathionate broth 25 per cent were positive.

### *Cultural Characteristics*

The 454 cultures of *Proteus* grew readily on standard agar and other mediums at 30 to 37 C. On EMB agar (1.3 per cent agar) *P. vulgaris* and *P. mirabilis* colonies possessed white, pink, or blue centers with the spreading growth a white to whitish blue color in 24 to 48 hours at 37 C. On SS agar (1.35 per cent agar) swarming was completely inhibited, even with moist surfaces. Well-isolated colonies of *P. vulgaris* and *P. mirabilis* had dark brown centers with white entire edges in 24 hours at 37 C. At 48 hours the centers became jet black,

but some isolated colonies failed to develop black centers in 3 to 4 days. Where colonies were crowded, 3 to 4 days elapsed before an occasional colony developed black centers. A strong odor of hydrogen sulphide was noted upon removal of the plate covers, and the removal of black-centered colonies revealed a black precipitate in the medium. On EMB agar *P. morganii* and *P. rettgeri* failed to spread in 24 hours at 37 C, but after an additional 24 hours some *P. morganii* strains showed spreading. Both species on EMB agar produced white, pink, or blue colonies. On SS agar colonies were colorless and opaque, and showed no spreading.

Almost without exception descriptions of the morphology of *Proteus* start with the phrase "highly pleomorphic rods." Most authors, however, fail to point out that pleomorphism in *Proteus* cultures is a transient characteristic and for the most part confined to actively swarming cultures. In smears from young cultures (5 to 7 hours' incubation) and young colonies (10 to 12 hours) pleomorphism is marked, but smears from the same culture or colony after 24 to 48 hours show little or no more morphological variation than comparable smears from the average coliform culture. On the other hand, smears, particularly from the periphery, of young colonies of an occasional coliform or paracolon culture may show pleomorphism almost as marked as does *Proteus*. In using pleomorphism as a criterion of *Proteus*, the age, motility, and swarming characteristics of the culture in question should be carefully recorded.

We have only one thing to add to the swarming characteristics of *Proteus* organisms as described by Hauser (1885), Moltke (1927), and others. On 1 per cent agar slants with water of expression in the base of the tube, straight line inoculations of *P. vulgaris*, *P. mirabilis*, and *P. morganii* behave similarly at 25 C. Growth along the line of inoculation is first established, then lateral, straight, or curved branches spread over the surface; often these branches reach the edge of the slant before the interstices are filled in. In such cases the line of inoculation can be distinguished easily from the swarm portion of growth. Frequently, however, the swarm growth is so thin that a needle is required to detect its presence. With *P. vulgaris* and *P. mirabilis*, especially the former, as the concentration of agar increases, the character of swarming is not markedly altered until it ceases. With *P. morganii*, as the concentration of agar increases, lateral branchings decrease in length until they disappear. The culture may continue to spread in a continuous wave outward from the line of inoculation to cover the entire surface of the slant. As the wave spreads, growth thickens until it is difficult to detect the line of inoculation. On very fresh 1 per cent agar slants a few strains of *P. rettgeri* show short lateral branches, but for the most part the culture spreads like *P. morganii* on higher concentrations of agar. On 1 per cent agar plates an occasional strain of *P. rettgeri* may swarm in a manner quite indistinguishable from *P. vulgaris*.

It is evident that the swarming of *P. vulgaris* and the spreading of *P. rettgeri* are quite different processes, but that they have a common origin is shown by the following fact. In experiments involving the growth curve of *P. vulgaris* several nonmotile strains were used. Occasionally one colony on several plates

used for counts showed some evidence of spreading. From a well-defined center an even ring of growth extended outward for several millimeters. No branches were observed from the edge of the colony. Transplants from the edge of the colony to 1.2 per cent agar slants exhibited spreading as described for *P. rettgeri*. With subsequent transplants spreading either ceased or by a series of transitional stages progressed to the characteristic swarming of *P. vulgaris*. Of the 69 *P. vulgaris* strains, 12, for the most part old stock cultures, were nonmotile. Three fresh isolates and one old stock culture were the only nonmotile strains of the 205 *P. mirabilis* strains studied. Of the 102 *P.morganii* strains, 2 fecal and 3 wound isolates were nonmotile. All *P. rettgeri* strains were motile, but many strains received by us were labeled "nonmotile." The motility of *P.morganii*, and particularly *P. rettgeri*, cultures is poor, or is completely inhibited, at 37 C.

### Biochemical Reactions

As previously pointed out, the 454 *Proteus* cultures used in this investigation, and over 200 additional *Proteus* cultures routinely recorded in this and neighboring diagnostic laboratories, produced acid only or from a bubble to 15 per cent gas in Durham fermentation tubes with any fermentable carbohydrate in 48 hours. With very few exceptions gas volumes were not appreciably increased on prolonged incubation. Of the 454 cultures studied in detail, only 3 strains of *P. mirabilis* produced as much as 20 to 30 per cent gas in sucrose in 3 to 5 weeks. Hereafter "the production of acid and gas" or a "+" reaction of *Proteus* in any carbohydrate will signify slight gas production, a bubble to 15 per cent gas. Gas production, especially in cultures from stock collections, is variable in sucrose, salicin, and maltose.

Biochemical reactions, characteristic of the different species, are given in table 1 and will be discussed for each species.

*Proteus vulgaris*. Of the 69 strains, 67 produced acid and gas in glucose in 24 to 48 hours. Two cultures gave acid only in 24 hours and no gas in subsequent tests after prolonged incubation. All strains produced acid or acid and gas in sucrose and maltose in 24 to 48 hours, though on occasions the reaction, particularly in maltose, was weak. Twenty-seven strains fermented salicin rapidly (24 to 48 hours), and 6 strains slowly (7 to 21 days). One strain fermented lactose and mannitol slowly. All strains hydrolyzed urea in 8 to 12 hours, produced hydrogen sulphide, and failed to produce acetylmethylcarbinol. Five failed to liquefy gelatin, 4 were indole-negative, and 35 were citrate-positive.

Recently isolated *P. vulgaris* strains differed considerably from those obtained from stock collections. Of 37 recent isolates, 4 per cent and, of 32 stock cultures, 87.5 per cent produced acid only in one or more of the carbohydrates, sucrose, salicin, and maltose. Nine recent isolates fermented salicin (3 rapidly and 6 slowly) for a total of 24.3 per cent, whereas 75 per cent of the stock cultures fermented salicin rapidly. Recent isolates liquefied gelatin in 1 to 3 days, 28 stock strains from 7 to 24 days, and 4 such strains were negative after 2 months. One recent isolate and 3 stock strains were indole-negative. (Contrary to the

statement [Bergey *et al.*, 5th ed.] that indole formation by *P. vulgaris* is slight, the positive strains gave an immediate and strong reaction with Kovac's reagent.) Twenty-five of the 35 citrate-positive strains were from stock collections and 5 of the 10 recent isolates required varying periods of time to grow on the medium. One stock culture produced acid in lactose and mannitol. Welch and Poole (1934) reported a variant of an X19 strain which fermented lactose and mannitol.

*Proteus mirabilis*. All 205 strains produced acid and gas rapidly in glucose. Acid or acid and gas was produced in sucrose slowly by 197 strains, and 19 of these strains also fermented salicin slowly. One otherwise normal strain produced acid slowly in lactose, and another otherwise normal strain produced acid and gas in maltose. The type of growth of *P. mirabilis* in sucrose broth fre-

TABLE 1

*Biochemical reactions characteristic of the different species of the genus Proteus*

	P. VULGARIS	P. MIRABILIS	P. MORGANII	P. RETTGERI
Strains studied . . . . .	69	205	102	78
Glucose . . . . .	+	+	+	A+
Lactose . . . . .	—	—	—	—
Sucrose . . . . .	+	+s	—	As
Salicin . . . . .	—+	—	—	—A+
Maltose . . . . .	+	—	—	—
Mannitol . . . . .	—	—	—	A+
Urea . . . . .	+r	+r	+s	+r
Hydrogen sulphide . . . . .	+	+	—	—
Gelatin . . . . .	+	+	—	—
Indole . . . . .	+	—	+	+
Citrate . . . . .	—+	+	—	+
Voges-Proskauer . . . . .	—	+—	—	—

s = slow.

r = rapid.

+ = acid and small gas volume in carbohydrates.

A = acid.

—+ indicates that a substantial number of strains fall in either category. For minor exceptions see section on biochemical reactions.

quently was a presumptive criterion for this species. It was characterized by a surface scum with marked sedimentation and decolorization of the brom cresol purple indicator after several days. All strains hydrolyzed urea rapidly. The Malay-Kingsbury HXK strain, though consistently positive, produced a nonmotile variant that was urea-negative. The ability of the variant to hydrolyze urea was restored after serial transplants in urea medium for 9 months. Two of the 205 strains failed to produce hydrogen sulphide. Recent isolates liquefied gelatin in 1 to 3 days except for 2 strains that required 10 to 14 days, whereas strains from stock collections, when positive, usually required 14 to 21 days. Seven stock strains, including 4 XK strains, failed to liquefy gelatin. Indole was formed by only 3 strains and one of these gave a weak reaction. Acetylmethylcarbinol was produced by 119 strains. The Voges-Proskauer



reaction was never strong, occasionally moderate, usually weak, and sometimes to be described as a trace. Furthermore, in a subsequent test 2 to 3 years later, 10 strains previously positive were negative. Nine strains failed to grow on citrate agar.

*Proteus morganii*. The biochemical reactions of this species were uniform. The 102 strains produced acid and gas rapidly from glucose. Sucrose was fermented slowly by 11 strains. Lactose, salicin, maltose, and mannitol were not attacked. One otherwise normal strain failed to form indole. Hydrogen sulphide and acetylmethylcarbinol were not produced, gelatin was not liquefied, and growth did not occur on citrate agar. All strains hydrolyzed urea in 24 to 48 hours. Levine *et al.* (1925), Rauss (1936), and others had reported *P. morganii* as producing hydrogen sulphide. Sevin and Battiaux (1939) observed only slight blackening on lead acetate with some strains in 4 to 6 days, whereas other strains showed no blackening. The discrepancy between our findings and those cited above is probably due to the fact that only definite blackening with diffusion into the medium was called a positive reaction in this work.

The growth of all strains of *P. morganii* in tryptone broth (distilled water containing 1 per cent bacto-tryptone) in 4 to 6 hours yielded a strong fetid odor; in 18 to 24 hours the odor was considerably diminished or even absent. Tribondeau and Fichet (1916) noted a fecal odor with this species in peptone broth. The rapid development of the odor proved to be of marked differential value for *P. morganii*.

*Proteus rettgeri*. Seventy-one of the 78 strains produced acid, and 7 produced acid and gas rapidly in glucose. All but 3 strains produced acid in 7 to 40 days in sucrose. Twenty-six strains produced acid and one acid and gas in salicin in 1 to 21 days. Seventy-one produced acid and 7 acid and gas rapidly in mannitol. Lactose and maltose were not attacked. Three strains failed to hydrolyze urea rapidly. All strains were indole-positive and grew on citrate agar. Hydrogen sulphide and acetylmethylcarbinol were not produced, and gelatin was not liquefied.

There remain to be described briefly 74 cultures, without taxonomic status, which biochemically resemble *Proteus*. Twenty-three of these cultures were previously described as type 29911 (Stuart, Wheeler, *et al.*, 1943). Seventy-one of the cultures were isolated from feces and 3 from urine. Acid or acid and gas was produced rapidly in glucose by all strains. Gas, when present, was limited to a small bubble. All strains produced acid in sucrose in 5 to 15 days and a weak acid reaction in maltose in 14 to 30 days. Lactose, salicin, and mannitol were not attacked. Sixty-seven strains did not hydrolyze urea. Seven strains hydrolyzed urea weakly, raising the pH to 7.2 or 7.4 in 48 hours without further increase even after 5 days (Rustigian and Stuart, 1941). All strains were indole- and citrate-positive, and gelatin-, acetylmethylcarbinol-, and hydrogen-sulphide-negative.

### *Serological Reactions*

Final detailed antigenic analyses of *P. vulgaris* must await further investigation. By H and O agglutination and adsorption test in antisera prepared

from 6 *P. vulgaris* strains, however, 42 of the 57 strains were divided into 5 groups, which were further subdivided into from 1 to 5 types in each group on the basis of possessing 2 or more major antigens in common. Of the remaining 15 strains, 13 showed only one major or minor antigen, and 2 had no antigens in common with any of the 5 groups. (Group as used in this report does not signify a number of antigenically identical cultures but rather a number of cultures with the same antigenic pattern when tested in two or more antisera.) Antigenically identical strains were relatively common among the X strains, but very uncommon among the others. The fact that further work is contemplated on this species does not imply that we hope to devise a practical system for

TABLE 2

*Representative antigenic groups of P. mirabilis as determined by cross-agglutination reactions of 138 strains of this species in four P. mirabilis antisera*

GROUP	STRAINS	ANTISERUMS			
		48	94	225	248
1	49	2,560	2,560	20,480	20,480
2	9	2,560	2,560	20,480	2,560
3	8	20,480	20,480	20,480	20,480
4	11	20,480	2,560	2,560	20,480
6	5	20,480	20,480	2,560	2,560
9	3		2,560	2,560	2,560
10	3				2,560
12	2	2,560	2,560	2,560	160
13	3		2,560	2,560	10,240
14	4	640	640	10,240	640
15	1	640		10,240	2,560
17	1		640		2,560
21	1			640	10,240
23	1	10,240	2,560		2,560
24	1			10,240	2,560
26	1	640	640	640	640
27	1	640	640		2,560
33	1	20,480	20,480	10,240	
38	2				

antigenically typing strains of *P. vulgaris*, for we are firmly convinced that this is impracticable.

To determine the serological relationships of *P. mirabilis* within the species, 138 strains were tested in 4 *P. mirabilis* antisera. Because of the number of tests involved, agglutination tests were set up with saline suspension of living organisms in antiserum dilutions of 160, 640, 2,560, 10,240, and 20,480. The 4 antisera possessed titers of 20,480. Adsorption experiments were done in a 160-dilution of the antisera. Only 2 of the 138 strains failed to agglutinate in any of the 4 antisera. The 138 strains could be divided into 38 groups on the basis of cross-agglutination reactions, the largest group containing 49 strains and the smallest 1 strain. Representative groups are shown in table 2.

Cultures agglutinating to titer or within one tube of titer in one or more antisera were used for adsorption.

Antiserum 48 was adsorbed with 25 strains, antiserum 94 with 23 strains, antiserum 225 with 85, and antiserum 248 with 80 strains. Four cultures reduced the titer of antiserum 48 from 20,480 to 1,280, 5 to 5,120, and 16 failed to reduce the titer. One culture completely adsorbed antiserum 94, 17 strains reduced the titer from 20,480 to 2,560 or 5,120, and 5 failed to alter the titer. No significant reduction in titer was caused by the 85 strains employed to adsorb antiserum 225. Two cultures completely adsorbed antiserum 248, two reduced the titer from 20,480 to 1,280, ten to 5,120, and sixty-six failed to reduce the titer. With a total of 213 adsorptions only 3 cultures were found homologous to one or another of the 4 cultures used to produce antisera.

TABLE 3

*Antigenic groups of P. morganii as determined by cross agglutination of 75 strains of this species in four P. morganii antisera*

GROUP	STRAINS	ANTISERUMS			
		213	278	305	312
1	8	20,480	20,480	640	
2	3	20,480	20,480	640	20,480
3	15	20,480	20,480	20,480	20,480
4	2	160	10,240	40,960	10,240
5	1	40	640	20,480	10,240
6	36		1,280	40,960	40,960
7	1	20,480	20,480	20,480	
8	1	640	1,280	10,240	5,120
9	1		10,240		160
10	1			10,240	5,120
11	1				20,480
12	1				2,560
13	1	640	160		160
14	1			640	160
15	2				

One of the 49 cultures in group 1, table 2, was used to prepare an antiserum. Of the 48 group 1 strains (exclusive of the strain homologous to the antiserum), 47 cultures agglutinated to titer, 40,960, or to within one tube of titer, and one to 640. Adsorption of the antiserum with the 48 cultures failed to reveal any culture identical with that used to produce the antiserum. Eight strains reduced the titer to 320 to 640, 18 strains to 1,280, 19 strains to 2,560 to 5,120, and 3 strains failed to reduce the titer. These and other experiments not herein recorded show the great antigenic complexity of *P. mirabilis*.

*P. morganii*, like *P. vulgaris* and *P. mirabilis*, lacked serological homogeneity. Seventy-five strains were tested in antisera prepared for 4 *P. morganii* strains, 213, 278, 305, and 312. The titers of these antisera were 20,480, 20,480, 40,960, and 40,960, respectively. Table 3 reveals 15 antigenic types as shown

by cross-agglutination titers in the 4 antisera. All strains agglutinating to titer or within one dilution of titer were used to adsorb the one or more antisera in which this reaction occurred. Antiserum 213 was adsorbed with 27 strains. Four group 1 strains (exclusive of 213) and three group 2 cultures completely removed the homologous agglutinins from antiserum 213. However, the group 2 strains and 213 were not identical since the former agglutinated to a titer of 20,480 and the latter failed to agglutinate in antiserum 312 (table 3). Some of the adsorbing strains reduced the titer of 213 antiserum to varying titers, whereas others did not alter the titer. One group 3 strain, exclusive of 278, completely removed the homologous agglutinins from antiserum 278, whereas 29 other adsorbing strains gave varying results. Of 20 used to adsorb 305 antiserum, one group 4 culture, exclusive of 305, completely removed homologous agglutinins, and the others varied in their reactions. Antiserum 312 was adsorbed with 42 strains; varying reactions were obtained, but none completely removed the homologous agglutinins.

The adsorption experiments above and others, such as the adsorption of antisera 305 and 312 with one of the 36 strains of group 6 and the testing of the 35 remaining strains, revealed a minimum of 30 major and minor antigens in the 75 *P. morganii* strains studied.

As with *P. vulgaris*, detailed antigenic analysis of *P. rettgeri* must await further investigation. Rustigian and Stuart (1943a) pointed out the serological heterogeneity of *P. rettgeri*. This was further confirmed in the present work by the study of 53 strains in 4 *P. rettgeri* antisera. A recent development, although not altering our concept of the antigenic heterogeneity of *P. rettgeri*, may modify the extent of heterogeneity within the species. In the early work on *P. rettgeri* (then called "anacrogenic paracolon, type 33111," Stuart, Wheeler, *et al.*, 1943) it was found that a strain not agglutinating at all in an antiserum could completely remove all agglutinins from the antisera upon adsorption. As the number of strains and antisera increased, this fact was ignored and the conventional procedure of testing *P. rettgeri* in the different antisera and adsorbing only with strains agglutinating to high titers was carried out. At the end of the present work the number of stock strains of *P. rettgeri* was reduced to 10 and the number of antisera to 3. Recently when heating several strains of *Shigella alkalescens* for rapid tube agglutination tests (Stuart, Rustigian, *et al.*, 1943) 4 cultures of *P. rettgeri* not agglutinating in the 3 antisera were also heated to 85 C for 1 hour. One strain agglutinated to titer in one antiserum and to partial titer in another, 2 strains agglutinated to partial titer in one or more antisera, and the other strain failed to agglutinate in any antiserum. The 3 antisera were adsorbed with the 4 cultures. The one culture agglutinating to titer completely removed the agglutinins from the serum, whereas the titer was not altered by any other strain upon adsorption. Tests in the 3 antisera of the 10 stock strains and 5 additional isolates after heating revealed, even with this small number of cultures and antisera, too great an antigenic heterogeneity for the practical typing of the species.

Of the type 29911 cultures, 7 were chosen to prepare antisera. One culture

previously reported (Stuart, Wheeler, *et al.*, 1943) agglutinated to titer in one antiserum and upon adsorption reduced the homologous titer from 20,480 to 320. Twenty-four cultures agglutinated to titers of 40 to 1,280 in one or another of the 7 antisera, whereas 41 failed to agglutinate in any antiserum. Varying numbers of *P. vulgaris*, *P. mirabilis*, *P. morganii*, and *P. rettgeri* cultures selected at random were tested for cross agglutination in the antisera of the other 3 species. Between *P. vulgaris* and *P. mirabilis*, and between *P. vulgaris* and *P. morganii*, common major and minor antigens were encountered in a large majority of the tests. On the other hand, major or minor antigens common to *P. mirabilis* and *P. morganii* were found in a minority of the tests. For example, of 132 *P. mirabilis* strains tested in 4 *P. vulgaris* antisera, every culture agglutinated to titers of 40 to 5,120 in one or another of the antisera, whereas of 100 of the same cultures, 74 failed to agglutinate in 3 *P. morganii* antisera, 12 agglutinated to a titer of 40, and 14 to a titer of 1,280 in one or another of the antisera. Twenty-two *P. rettgeri* cultures were tested in 4 *P. vulgaris*, 2 *P. mirabilis*, and 4 *P. morganii* antisera. Three strains agglutinated to 640 in one *P. vulgaris* serum, and to 1,280 in one *P. mirabilis* serum; 14 other strains to titers of 160 to 320 in three of the four *P. morganii* antisera. Of 36 type 29911 strains tested in 4 antisera each of *P. vulgaris*, *P. mirabilis*, and *P. morganii*, 19 agglutinated to titers of 80 to 1,280 in one or another of the 12 antisera.

Antigenic relationships between coliform, paracolon, and *Salmonella* cultures have been found by Schiff *et al.* (1941), Stuart, Wheeler, *et al.* (1943), Wheeler *et al.* (1943), and others. Bornstein *et al.* (1941) reported antigens common to *Salmonella* and *Shigella* cultures. *Proteus* cultures with antigens in common with *Shigella* have been discovered. A culture of *P. morganii* received in this laboratory agglutinated strongly in pooled *Salmonella paratyphosa* antiserum. Wheeler (personal communication) found that this culture possessed the specific antigen of *S. paratyphosa* type III. Other tests in this laboratory and in the Bureau of Laboratories of the Connecticut State Department of Health have revealed several minor antigens common to *Proteus* and *Shigella* cultures.

#### DISCUSSION

Despite the fact that as early as 1893 Smith recognized the "peculiar" nature of gas production by *Proteus* cultures most investigators have used as the principal test for inclusion in this genus fermentation reactions in glucose, lactose, and sucrose without reference to gas volume. This oversight has permitted strains of paracolon bacteria to be classified as members of the genus *Proteus*. Moreover, failure to recognize that sucrose fermentation by *P. mirabilis* may be slow and that exceptions may occur in the biochemical reactions of almost any species of bacteria has led to giving specific rank to variants of one species.

St. John-Brooks and Rhodes (1939), Reed and Tonner (1942), Rustigian and Stuart (1941, 1943a), Thornton (1944), and others would exclude *Proteus hydrophilus* and *Proteus ichthyosmii* from the genus *Proteus*. Rustigian and Stuart (1941), except for gelatin liquefaction, failed to find any characteristics in common

between *Proteus bombycis* and the genus *Proteus*. This was further confirmed in the present investigation on five strains each of *P. hydrophilus* and *P. ichthyosmius* and one of *P. bombycis*. All the foregoing investigators who have worked on *Proteus americanus* and *Proteus ammoniae* agree that these are variants of *P. mirabilis*. Of special interest is the work of De Assis (1939) who demonstrated increasing acid and gas production with serial transplants of *P. americanus* in sucrose medium. A strain of *Proteus pseudovaleriei* from the American Type Culture Collection possessed few of the characters noted for this organism in Bergey *et al.*, 5th ed., but did possess the characteristics of *P. mirabilis*. A culture of this organism received from De Assis agreed with the description in Bergey *et al.*, 5th ed., but failed to swarm under any conditions, produced relatively large gas volumes in all fermentable carbohydrates except lactose, and did not attack urea. This culture had all the characteristics of a paracolon organism.

The careful and detailed analyses of *Proteus melanovogenes* made by Miles and Halnan (1937) reveals that by present-day standards this organism should not be in the genus *Proteus*. While actively motile, it is monotrichate. Attempts to produce the peritrichous condition by twice-daily transplants for 3 weeks in 0.3 per cent semisolid agar failed. *P. melanovogenes* produced acid and gas "vigorously" in glucose, sucrose, and maltose in one day, and in mannitol in one or two days. Some strains attack lactose slowly and weakly, and some strains attack salicin in the same way. This organism did not swarm, gelatin liquefaction was slow, and urease was not produced. In fairness to these investigators it should be pointed out that they recognized the limitations of their cultures and the organism was "tentatively" classed in the genus *Proteus*.

Steinhaus (1941) classified 3 organisms isolated from insects in the genus *Proteus*. An attempt to obtain these cultures failed since by the time the paper was published the cultures had been discarded. The criteria for the genus *Proteus* for these organisms appears to have been no action on lactose, liquefaction of gelatin, and "slight production of hydrogen sulfide" for 2, and gelatin liquefaction for the other. The 3 cultures, although motile, did not swarm or spread according to the description of agar colonies. Two cultures were indole-negative and fermented both maltose and mannitol, which would exclude them from any recognized species in the genus. Moreover, the fermentation of both mannose and rhamnose would exclude them from the genus (Moltke, 1927). The remaining culture was indole-negative and also fermented maltose. Only 4 carbohydrate reactions were recorded for this culture. Urease production was not determined. It is highly probable that all 3 cultures in question were members of the intermediate group of the paracolon since some strains of the group are indole-negative, liquefy gelatin, and produce hydrogen sulphide (Stuart, Wheeler, *et al.*, 1943).

Lobik (1915) described *Proteus nadsonii* as a peritrichous rod, liquefying gelatin, producing hydrogen sulphide, not forming indole, coagulating but not peptonizing litmus milk, and having a variable gram stain. No carbohydrate reactions were given. As this organism was indole-negative, if in the genus

*Proteus* it would have to be *P. mirabilis* (or by the British system *P. vulgaris*), but both these species peptonize milk readily (Bergey *et al.*, 5th ed.) Moreover, the variable gram stain would seem to exclude this organism from the genus *Proteus*.

Three organisms labeled with the epithets *diffluens*, *metadiffluens*, *paradiffluens* were described by Castellani and Chalmers (1919) as species of the genus *Proteus*, primarily on the basis of gelatin liquefaction. *P. metadiffluens* is clearly not in the genus *Proteus* as it does not ferment glucose, sucrose, or maltose. *P. diffluens* differs from *P. mirabilis* in its failure to ferment sucrose and peptonize litmus milk. Litmus milk was not used, but it was found that one strain of *P. mirabilis* required 5 weeks to ferment sucrose, and 2 sucrose-negative strains were encountered. *P. paradiffluens* appears to be a normal *P. mirabilis* including fermentation of sucrose and peptonization of milk. The *Proteus speudomorganii* of Castellani and Chalmers (1919) is a normal *P. morganii*, whereas their *P. morganii* is an O form of the same species.

The *Proteus sp. nov.* reported by Warren and Lamb (1924) was isolated from blood of a patient eleven days before death. The organism produced acid and gas in glucose, sucrose, maltose, and mannitol, and acid only in salicin. Although motile, colonies of the organism did not "spread," gelatin was liquefied, indole was positive, and milk was coagulated and peptonized. There was "slight browning in lead acetate." Since fermentation reactions were observed for only 7 days, this organism, fermenting both maltose and mannitol, could have been a slow lactose-fermenting paracolon.

*Proteus photuris* (Brown, 1927) was isolated from the luminous organs of the firefly (*Photuris pennsylvanicus*). The organism was a "minute," nonmotile, gram-negative, "bipolar," staining rod. Thirty-day-old colonies were "very irregular, amoeboid, slimy growths, white at the margin and yellowish to light brown in the middle." Gelatin was liquefied but there was no digestion of milk. Hydrogen sulphide was not produced. Acid and gas were formed in glucose, sucrose, and mannitol. Gas volumes in these carbohydrates could have been moderate to large since it is stated that gas was weakly produced in inulin. Aberrant coliforms, slow or nonfermenting lactose strains, with the colonial and biochemical characteristics of *P. photuris*, have been studied in this laboratory. These cultures were isolated from water, soil, and cereal and were classed as aberrant *Aerobacter* (Stuart, Mickle, and Borman, 1940).

Reference must be made also to the organisms described as *P. morganii* types by Waaler (1931) and others based on the work of Morgan and Ledingham (1909). With difficulty 4 cultures tentatively classified as *P. morganii* types XII and XIV were obtained. On the basis of relatively large gas volumes, inability to swarm though motile, and inability to produce urease, these organisms were considered to be paracolon cultures. Waaler's (1931) five *P. morganii* type XII strains, from their characteristics, appear to be members of the paracolon group. Since the term *P. morganii* is now used to designate a species, it is recommended that it no longer be employed for other organisms. Such practices connote a close relationship to *P. morganii* and add confusion to the concept of the genus *Proteus*.

The four species of the genus *Proteus* described in the present investigation constitute a fairly compact group of organisms. Urea hydrolysis and failure to ferment lactose appear to be the best criteria for the genus. More than 600 *Proteus* cultures have been tested in the buffered urea medium described by Rustigian and Stuart (1941). All cultures were positive except 5: one was a urea-negative variant of a urea-positive parent; one strain of *P. mirabilis* from a stock collection, although positive when received, was negative 4 years later; one *P. rettgeri* strain was completely negative; and 2 others were only moderately positive (pH 7.4 to 7.8). A subsequent publication will show that in the family *Enterobacteriaceae* utilization of urea is not restricted to *Proteus* organisms even though evidence of hydrolysis in the buffered medium cited is limited to the genus *Proteus*. Gas production is an excellent criterion for 3 species of the genus. Relatively recent isolates of *P. vulgaris*, *P. mirabilis*, and *P. morganii* almost without exception produce small gas volumes in all fermentable carbohydrates even after prolonged incubation. Many old stock cultures of these species were found to be anaerogenic. Most strains of *P. rettgeri* were anaerogenic; an occasional strain produced a small amount of gas. Swarming under suitable conditions is an excellent criterion for H forms of 3 species, but is without value for the O forms of these 3 species and for some strains of *P. rettgeri*. Gelatin liquefaction can no longer be accepted as a characteristic of the genus though it may serve to differentiate species within the genus. This also holds true for production of indole and hydrogen sulphide and for growth on citrate agar.

From an academic viewpoint there is adequate evidence for differentiating *P. vulgaris* and *P. mirabilis*. The former characteristically acts on glucose, sucrose, and maltose rapidly and produces indole; whereas *P. mirabilis* characteristically acts on glucose rapidly, sucrose slowly, and not at all on maltose, is indole-negative, and produces acetylmethylcarbinol in many instances. Furthermore, *P. vulgaris* is Eijkman negative, whereas *P. mirabilis* is positive (Stuart, Zimmerman, *et al.*, 1942; Stuart, Van Stratum, and Rustigian, 1944). On the other hand, in the British system these 2 species are united under *P. vulgaris*. The swarming characteristics and the ability to liquefy gelatin, to produce hydrogen sulphide, to grow on citrate agar, and to produce urease rapidly show the close relationship of these two "types." The fact that only 10 to 20 per cent of the isolates of the two "types" are *P. vulgaris* may indicate that the minority group are variants of the majority group. Moreover, an occasional strain is isolated possessing characteristics intermediate between the two "types." The present authors are not in perfect agreement on this point. One of us (Rustigian) is inclined to give specific rank to both *P. vulgaris* and *P. mirabilis*. The other (Stuart) is inclined to group the two "types" into one species, *P. vulgaris*, for the purpose of taxonomic simplification (Borman *et al.*, 1944).

*P. morganii* seems clearly established as a member of the genus *Proteus*. It utilizes urea more slowly than the other species in the genus. *P. rettgeri* also seems clearly established in the genus despite its borderline characteristics. As previously pointed out, many strains spread on moist 1 per cent agar plates but only an occasional strain covers the entire surface of the agar. Two strains



attacked urea only moderately and one not at all. All strains reduced trimethylamine oxide weakly. Two strains were negative on one test but were weakly positive on subsequent tests. Other *Proteus* strains reduce the compound strongly and rapidly (Wood and Baird, 1943; Wood, personal communication). Recently, Wheeler tested 250 *Proteus* cultures for trimethylamine oxide reduction and found that 6 cultures were unable to reduce this compound (personal communication).

Biochemically and antigenically, organisms of the genus *Proteus* present a much more compact picture than do those of the coliform group. Common antigens are relatively infrequent among coliform bacteria as a whole (Stuart, Baker *et al.*, 1940). In *Aerobacter* antisera 16.9 per cent of *Aerobacter* strains agglutinated to an average titer of 1,148; in intermediate antiserum 7.4 per cent of the intermediate strains reacted to an average titer of 1,572; and in *Escherichia* antisera 38.4 per cent of the *Escherichia* strains agglutinated to an average titer of 1,261. With *Proteus* cultures, each in its own antiserum, 66.5 per cent of *P. vulgaris*, 92.6 per cent of *P. mirabilis*, 78 per cent of *P.morganii*, and 59 per cent of *P. rettgeri* strains agglutinated to average titers of 13,726, 7,804, 12,779, and 4,725, respectively. (A higher percentage of cultures agglutinating and a higher average titer would probably have resulted from the use of heated antigens with *P. rettgeri*.) The marked antigenic continuity of antigens in the species of *Proteus* is not indicative of great numbers of antigenically identical strains within the different species. Except for X strains, antigenically identical strains are relatively infrequent within each species. Common antigens among the different species are frequently encountered. Antigenic typing as with the *Salmonella* group would be a tremendous task incommensurable with the practical importance of the genus *Proteus*.

#### CONCLUSIONS

The genus *Proteus* is a relatively homogeneous group biochemically, and on this basis can be divided into four species—*P. vulgaris*, *P. mirabilis*, *P.morganii*, and *P. rettgeri*.

The first two species could be combined into one, *P. vulgaris*, as in the British system for taxonomic simplification.

Urease production for all species and small gas volumes, except with *P. rettgeri* which usually produces acid only, are two cardinal physiological criteria of the genus. Gelatin liquefaction and fermentation of glucose and sucrose but not lactose do not satisfactorily delineate the genus *Proteus*.

The genus *Proteus* is serologically heterogeneous, although common antigens are frequently encountered among the different species.

It is recommended that *Proteus ammoniae* and *Proteus americanus* be considered as variants of *P. mirabilis*, and that *Proteus hydrophilus*, *Proteus ichthyosmius*, *Proteus pseudovaleriei*, and *Proteus bombycis* be excluded from the genus *Proteus*.

It is recommended that the epithet "morganii" be employed solely for *Proteus morganii* (Morgan's bacillus number 1) as described by Morgan and Ledingham.

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# FURTHER STUDIES ON UREASE PRODUCTION BY PROTEUS AND RELATED ORGANISMS

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In 1941 Rustigian and Stuart reported a new medium for the detection of urease production by organisms of the genus *Proteus*. These investigators tested 151 *Proteus* cultures in the medium and all were positive. One urea-positive X strain, however, did produce a urea-negative variant, which after prolonged cultivation in urea medium regained its ability to produce urease. Using this medium Ferguson and Hook (1943) found that 29 cultures with the biochemical reactions of *Proteus* produced urease, whereas 75 *Salmonella* cultures did not. This urea medium, which contains the hydrogen ion indicator, phenol red, demonstrates production of urease by a decrease in the hydrogen ion concentration. *Proteus vulgaris* and *Proteus mirabilis* gave a positive reaction, pH 8.1 or more, in about 8 hours, whereas *Proteus morganii* required about 36 hours. Rustigian and Stuart (1943) found that, except for one strain, *Proteus rettgeri* attacked urea rapidly in about 12 hours.

To date in this laboratory over 600 *Proteus* cultures have been tested in the urea medium. In addition to the two exceptions noted above, one old stock culture, positive when first received, became urea-negative four years later, and two strains of *P. rettgeri*, which on first test produced only weak reactions, pH 7.2 to 7.4, in 48 hours after serial transfers in the medium were positive in 12 hours. An occasional freshly isolated strain of *P. rettgeri* may require as much as 18 to 24 hours to give a positive reaction.

Rustigian and Stuart (1943) tested in their medium 689 cultures representing all genera in the family *Enterobacteriaceae* exclusive of *Proteus*. A number of coliform cultures, particularly *Aerobacter* strains, attacked urea slowly and weakly, pH 7.0 to 7.2 in five days. To date an additional 418 cultures representing all genera in the family *Enterobacteriaceae* except *Erwinia* and exclusive of *Proteus* have been tested. A number of coliform cultures attacked urea slowly and weakly, whereas several cultures (to be described later in this report) gave a strong reaction in 4 to 7 days. Thus, in tests on over 1,000 cultures in the family *Enterobacteriaceae* other than *Proteus* no culture was positive in the time prescribed for *Proteus*, 8 to 48 hours.

## *Standard Urease Test*

In this laboratory the urea medium is usually made up in lots of 400 ml since this amount is contained in the 5" x 1" filter candle and mantle used to sterilize the medium. The medium is made as follows: To 380 ml of distilled water are added 3.64 grams of  $\text{KH}_2\text{PO}_4$ , 3.8 grams of  $\text{Na}_2\text{HPO}_4$  (Sorensen buffers), 8 grams of urea (highest purity), 40 milligrams of yeast extract (Difco), and 20 ml of

a 0.02 per cent solution of phenol red. This highly buffered medium has a pH of 6.8. The medium is tubed in approximately 3-ml amounts in tubes 14 mm inside diameter and 125 mm long. Inoculations are made from 18- to 24-hour agar slant cultures with a straight needle, and tests are incubated at 37 C in an air incubator. When possible, reactions are recorded after 8, 12, 24, and 48 hours of incubation. In the previous reports on this medium no particular conditions of the test were given since conditions were kept constant and no unusual reactions were encountered.

From different laboratories an occasional *Proteus* culture was received which was reported to be either faster or slower in attacking urea than the standard set for the particular species. These cultures proved to be quite normal when tested in this laboratory. Further inquiry revealed in every case some variation from the procedure used in this laboratory, such as inadequate buffering of the medium, temperature variations, incubation at 37 C in air incubators and water baths, and, to conserve medium, tubing in small serological tubes. When conditions reported by different laboratories were duplicated, their results were confirmed. It seemed advisable to test carefully the conditions surrounding the production of urease by *Proteus* and related organisms. In order to conserve medium 1.5 ml of medium in 14 x 125 mm tubes, the smallest amount that could be read accurately in a comparator, was accepted as standard. When comparative inoculums were needed, 5 ml of sterile saline were added to a 24-hour agar slant culture; the organisms were suspended by rolling the tube briskly between the hands, and inoculations in the desired quantities were made with sterile pipettes. When accurate amounts were required, the medium was pipetted into sterile plugged tubes with sterile pipettes. A reaction was recorded as positive when the pH was 8.1 or more.

#### *Size of Inoculum, Amount of Medium, and Aeration*

To a series of 15 tubes containing 1.5 ml of medium, 0.01 ml of a bacterial suspension was inoculated into the first, 0.02 into the second, 0.03 into the third, etc., up to 0.1, then 0.2, 0.3, 0.4, and 0.5. After 4 hours' incubation at 37 C tubes inoculated with the rapid urease-producing species (*P. vulgaris*, *P. mirabilis*, and *P. rettgeri*) were read every half hour, whereas with the slow urease-producing species (*P. morganii*) readings were made at hourly intervals beginning with the eighteenth hour. As the size of the inoculums increased from 0.01 to 0.1 ml, the time required for the rapid species to reach pH 8.1 decreased, but there was no further acceleration with heavier inoculums. The 0.1 inoculums were positive on an average of three hours earlier than the 0.01 inoculums. On the other hand, no significant alteration in the time required for *P. morganii* to become positive was noted for any of the inoculums. One-tenth milliliter was accepted for the standard inoculum.

When constant inoculums (0.1 ml) were made into tubes containing 1.5, 3, 4.5, and 6 ml of medium, the time required for a positive reaction increased with the volume. With the rapid urease-producing species the smallest volume was positive in an average of 4.5 hours before the largest, and the difference with

*P. morganii* was about 12 hours. Since these differences seemed to be due to the ratio of surface area to volume of medium, the following tests were made. Two milliliters of the medium were placed in tubes with inside diameters of 9, 13, 18, and 24 mm, and the standard inoculum was added. With the rapid species a positive reaction was obtained in the 24-mm tube on an average of 3.5 hours before the 9-mm tube, but with *P. morganii* the difference in the speed of a positive reaction between these tubes was 18 hours.

Two sets of tubes with the standard amount of medium and inoculum were prepared from the rapid urease-producing species. One set was shaken every 30 minutes. The shaken tubes were positive on an average of 1 hour and 30 minutes before the unshaken. Except for *P. morganii*, variations in the time required to produce a positive reaction with alterations in the conditions of the test are not very great.

### Temperature

With changes in temperature, however, changes in the rate of urease production are so great, particularly for *P. morganii*, that at certain temperatures it cannot be distinguished from the other species. For the experiments on temperature two water baths that were regulated to  $\pm 0.1$  C were employed. Because of the number involved, straight needle inoculums from 24-hour agar slant cultures were made into approximately 1.5 ml of medium. Tubes were inoculated in groups of 6 and immediately placed in the water bath. The surface of the urea medium in the tube was 2.5 inches below the surface of the water. Twenty-four cultures each of *P. vulgaris*, *P. mirabilis*, and *P. rettgeri*, and 48 strains of *P. morganii* were tested at 20, 30, 35, 37.5, 40, 42.5, and 45 C. For the 37.5 C incubation, two tubes of urea medium were inoculated from each culture; one set of tubes was incubated in the water bath, the other in an air incubator. Results were recorded every 2 hours throughout an experiment. The results of these tests are shown in table 1.

Table 1 shows that as the temperature increases from 20 to 37.5 C in the water bath, the speed of urease production increases for *P. rettgeri*, but the speed was actually greater in the air incubator than in the water bath at 37.5 C. As the temperature continued to increase, the speed was decreased until at 45 C no strain was positive; 15 strains showed pH reactions of 7.0 to 7.2, and 9 strains produced no change from the original pH of 6.8. Essentially the same pattern was established by *P. vulgaris* except in the time involved, and in the reactions at 45 C. At 45 C the first strain was positive in 8 hours, 12 others in 24 hours, whereas 11 strains showed reactions varying from pH 6.8 to 7.5 in 24 hours, with no further changes in 48 hours. *P. mirabilis* is less affected by the different temperatures than the other species. No difference was noted in the speed of urease production at 37.5 C in the water bath and air incubator; the greatest speed was shown at 40 C with only slight decreases at 42.5 C and 45 C. Such reactions at 45 C were anticipated because Stuart, Zimmerman, *et al.* (1942) found *P. mirabilis* characteristically Eijkman-positive, whereas *P. vulgaris* and *P. morganii* were negative. The difference in the speed of urease production

by *P. morganii* at the different temperatures is very marked. At 20 C, the first, median, and last strains to go positive did so in 32, 58, and 130 hours, respectively, whereas at 42.5 C the same reactions took place in 4, 6, and 8 hours, respectively. The median strain at 37.5 C was positive in 10 hours in the water bath and 20 hours in the air incubator. Tubes in the water bath reached temperatures of 37.5 C in 5 minutes and 30 seconds, and in the air incubator in 2 hours and 15 minutes. The reaction of *P. morganii* at 45 C was not anticipated for reasons stated above.

When the experiment at 45 C was duplicated with inoculums from slants which had stood for approximately 2 months at room temperature, *P. rettgeri* and *P. vulgaris* and 15 strains of *P. mirabilis* failed to alter the pH of the medium. Nine strains of *P. mirabilis* ranged from pH 7.0 to 7.6. There was very little difference, however, in the speed of urease production by the young and old strains of *P. morganii*. Young strains usually became positive 2 hours before

TABLE 1

Time in hours required for the first culture, the median culture, and the last culture to become positive in urea medium (pH 8.1 or higher) for *Proteus* species at the various temperatures

INCUBATION .....		WATER BATH																								AIR		
Temperature .....		20 C				30 C			35 C			37.5 C			40 C			42.5 C			45 C			37.5 C				
Species	No.	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
<i>P. rettgeri</i> .....	24	14	22	30	8	14	22	6	12	18	6	12	14	8	12	14	10	24	58	a	a	a	6	10	14			
<i>P. vulgaris</i> .....	24	12	16	26	6	10	16	6	8	14	4	8	14	4	8	10	4	10	30	8	24	b	6	8	12			
<i>P. mirabilis</i> ....	24	12	18	22	6	8	10	4	8	10	4	6	6	4	4	6	4	6	6	4	8	10	4	6	6			
<i>P. morganii</i> ...	48	32	58	130	22	32	58	12	20	32	6	10	12	4	8	12	4	6	8	4	8	14	16	20	32			

A = first culture to become positive.

B = median culture, the 12th or 24th culture in each species.

C = last culture to become positive.

a. Nine strains produced no change in the medium while 15 gave reactions of pH 7.0 to 7.4.

b. Four strains produced no change in the medium while 7 gave reactions of pH 7.0 to 7.5.

the old strains, but, on the other hand, the last old strain to go positive did so in 12 hours, whereas the slowest young strain required 14 hours.

### Eijkman Reaction

In view of the unexpected reactions of *P. morganii* in urea medium at 45 C, it was decided to reinvestigate the Eijkman reactions of *Proteus*, including *P. rettgeri*, which was not tested in the previous work (Stuart, Zimmerman, *et al.*, 1942). Buffered glucose broth with brom thymol blue as indicator was used. Inoculations were made from agar slants (straight needle) and from broth cultures (2-mm loop). Cultures used for inoculations were 24 hours old. A total of 237 cultures, some of which were used in the previous work, were inoculated in groups of six and immediately placed in the 45 C water bath. (In the previous work a temperature of 45.5 C was used.) Recordings were made at 24 and 48

hours as no growth, growth, weak acid, and strong acid. As in the previous work, gas production was not recorded because many of the *P. mirabilis* strains produced no gas after 48 hours at 45 C. After 48 hours' incubation at 45 C. all tubes except those of *P. mirabilis* were incubated at room temperature and examined for 9 days for viability as shown by acid or acid and gas production.

The results shown in table 2 clearly confirm the work of Stuart, Zimmerman, *et al.* (1942) and in addition show that *P. rettgeri* is Eijkman-negative. They also show that agar slant inoculums of *P. morganii* uniformly grew at 45 C, but failed to attack glucose at the same temperature. However, these same strains produced urease strongly at this temperature. A greater number of *P. morganii* strains from agar slant inoculations were viable after 48 hours at 45 C than of *P. rettgeri* and *P. vulgaris*.

TABLE 2

*Eijkman reactions of 237 Proteus cultures including all species of the genus Proteus*

SPECIES	STRAINS	24 HOURS								48 HOURS								NO. OF CULTURES VIABLE AFTER 48 HOURS AT 45 C	
		Ag. inoc.				Br. inoc.				Ag. inoc.				Br. inoc.					
		-	G	A	A <sup>2</sup>	-	G	A	A <sup>2</sup>	-	G	A	A <sup>2</sup>	-	G	A	A <sup>2</sup>		
<i>P. rettgeri</i> . . . .	48	27	21	0	0	48	0	0	0	0	48	0	0	48	0	0	0	14	0
<i>P. vulgaris</i> . . .	46	14	31	1	0	45	1	0	0	0	45	0	1	45	0	1	0	5	1
<i>P. mirabilis</i> . . .	91					0	6	1	84					0	0	3	88		
<i>P. morganii</i> . . .	52	0	52	0	0	52	0	0	0	0	52	0	0	52	0	0	0	19	0

Ag. inoc. = agar inoculation.

Br. inoc. = broth inoculation.

- = no growth.

G = growth.

A = weak acid.

A<sup>2</sup> = strong acid.

### *Urease Production by Enterobacteriaceae*

The ability of other genera in the family *Enterobacteriaceae* (except *Erwinia*) to produce urease was investigated. By decreasing the concentration of buffers in the urea medium it was found that 0.01, the usual amount, would maintain a pH of 6.8 in nonpyrex culture tubes. Hereafter a batch of medium containing 0.01 of the original amount of buffers will be called a "0.01 buffered medium" and with 0.1, the original amount, a "0.1 buffered medium." Unless specified, all of the ingredients will be as in the original medium. These tests were incubated in a water bath at 37.5 C. To determine ammonia production from yeast extract alone a batch of 0.01 buffered medium without urea was prepared. In this medium 209 cultures representing all genera in the family *Enterobacteriaceae* and all important species in each genus (except *Erwinia*) were tested with agar slant inoculums. Every culture gave a pH reaction of 7.4 to 7.5 in 24 to 48



hours. In a 0.01 buffered medium containing urea 255 cultures exclusive of the genus *Proteus* were tested for urease production. Tests were read at 4, 8, and 12 hours, and at 12-hour intervals thereafter for a total of 48 hours. Twenty-six normal or paracolon *Escherichia*, 7 normal or paracolon intermediates, 44 normal or paracolon *Aerobacter*, 7 *Shigella alkaescens*, and one *Shigella equirulis*, or a total of 85 cultures, were positive (pH 8.1 or more) in one or two days. Although not positive, several additional coliform and 2 *Serratia* cultures probably would have been positive in another 24 or 48 hours. No *Salmonella* or *Shigella*, other than *S. alkaescens* and *S. equirulis*, showed any evidence of urea utilization. (These results confirm the work of Mitchell and Levine, 1938, White and Hill, 1941, and others, who showed that many coliform cultures, especially *Aerobacter*, may produce urease.) The 85 positive cultures were then tested in 0.1 buffered medium containing urea. After 24 hours' incubation the tubes were read at 12-hour intervals for 5 days. One paracolon intermediate, 24 normal or paracolon *Aerobacter*, and one *S. equirulis*, or a total of 26 cultures, were positive in 2 to 3 days. These 26 cultures were tested in standard urea medium. One paracolon intermediate, one *Aerobacter*, 8 paracolon *Aerobacter* type 32011 (Stuart, Wheeler, *et al.*, 1943; Stuart and Rustigian, 1943), and one *S. equirulis*, or a total of 11 cultures, were positive in 4 to 5 days.

It is evident that urease production is not confined to the genus *Proteus*, but the detection of urea utilization in the standard medium in 48 hours or less is confined to *Proteus* species. It is also evident that so far as their urease production is concerned, *Proteus* organisms are more closely related to *Aerobacter* than to other members of the coliform group. Fifty-four type 29911 cultures (Stuart, Wheeler, *et al.*, 1943; Rustigian and Stuart, 1944) were tested in 0.01 buffered medium. Twenty-six were positive in 12 to 24 hours. In the 0.1 buffered medium 15 were positive in 2 to 3 days. None were positive in the standard medium although 7 strains produced pH reactions of 7.2 to 7.6 in 5 days.

#### *Rapid Urease Test*

A rapid test for urease production by *Proteus* species, particularly *P. morganii*, would occasionally have marked diagnostic value in saving 24 to 48 hours. Straight needle inoculums into the 0.01 buffered medium failed since, although a majority of cultures of each species were positive in 2 to 4 hours, some required several hours for a positive reaction. Experiments varying the size of the inoculum yielded a satisfactory test though it did not differentiate between the rapid and slow urease-producing species of *Proteus*. The test consists of inoculating 3 loopfuls (approximately 2-mm loop) from an agar slant culture into the medium, shaking the tube, and, when necessary, incubating the tube in a 37.5 C water bath. A total of 225 *Proteus* cultures have been tested by this method. Readings were made at 5-minute intervals. Of 47 *P. vulgaris* strains the first was positive in 5 minutes, the median in 10 minutes, and the last in one hour and 20 minutes. Of 91 *P. mirabilis* strains the first was positive in 5 minutes, the median in 15, and the last in 60 minutes. Fifty-two *P. morganii* strains were

tested; the first was positive in 5 and all were positive in 15 minutes. Of 35 *P. rettgeri* strains the first was positive in 10 minutes, the median in 20, and the last in 60 minutes. Tubes were inoculated in groups of six. An occasional tube was actually positive by the time the tubes reached the water bath, but these were recorded as positive in 5 minutes. Such cultures were further studied. One of these, *P. mirabilis* 231, has been tested about 50 times, mostly in demonstrations; the fastest time for a positive reaction was 40 seconds and the longest was one minute and 15 seconds. The 255 cultures representing all genera of the family *Enterobacteriaceae* except *Erwinia* and exclusive of *Proteus* were tested by the rapid method. No culture was positive under 12 hours.

It will be recalled that in the regular medium at 45 C old agar and young agar slant inoculums of *P. morganii* showed little difference in the speed of the urease production, whereas only the young agar slant inoculums of *P. mirabilis* gave positive reactions. At the time the rapid test was perfected, there were 12 cultures each of *P. rettgeri*, *P. vulgaris*, and *P. mirabilis*, and 24 *P. morganii* about 6 months old. These were tested by the rapid method. Of the first 3 species mentioned the first culture was positive in 2 and the last in 5 hours. With *P. morganii* all but 6 of the 24 cultures were positive in 10 minutes, and these 6 were positive in 15 minutes.

#### DISCUSSION

It is evident from the foregoing that in strongly buffered urea medium only members of the genus *Proteus* give evidence of urea utilization. In weakly buffered medium, however, urea utilization is shown by many coliform and paracolon cultures, especially of the *Aerobacter* type. One normal *Aerobacter* strain and several paracolon *Aerobacter* type 32011 strains were capable of producing a positive reaction in regular urea medium in 4 to 6 days. One cardinal characteristic of type 32011 is the small gas volume produced in maltose and mannitol in 24 hours (Stuart, Wheeler, *et al.*, 1943). Recently from gastroenteritis patients several strains of type 32011 have been isolated which on first tests in glucose produced only a bubble of gas. In subsequent tests gas volumes increased in this carbohydrate to the usual 20 to 30 per cent volume.

It is interesting that *S. alkalescens* exhibited urea utilization in the 0.01 buffered medium. The biochemical and antigenic relationships of *S. alkalescens* to coliform, paracolon, *Shigella* (Stuart, Rustigian, *et al.*, 1943), and now in a small way to *Proteus*, constitute a remarkable range of characteristics.

The taxonomic position of type 29911 cultures is not clear. They bear some resemblance to *Proteus*: they produce acid or a bubble of gas in glucose, and acid and occasionally a bubble of gas slowly in sucrose; they have a weak acid reaction in maltose in 3 to 5 weeks; they produce indole; and they grow on citrate agar. A few strains "spread" on fresh moist agar (Rustigian and Stuart, 1944). Antigenically they are the most heterogeneous type or group of cultures encountered in this laboratory in an eight-year study on organisms of the family *Enterobacteriaceae*. Most cultures of this type were isolated from gastroenteritis patients. Several cultures of this type were received from other laboratories as

"*P. mirabilis*." The production of indole and failure to produce hydrogen sulphide (Rustigian and Stuart, 1944) would ordinarily exclude type 29911 from the species *P. mirabilis*, and their failure to attack urea readily from the genus *Proteus*. The taxonomic position of this type or group of organisms must depend on further work.

Urease production like most, if not indeed like every, biochemical test used to differentiate the various genera and species in the family *Enterobacteriaceae* shows no absolute line of demarcation, but rather a series of intergrading relationships among the different genera and species. These relationships can be studied best in weakly buffered urea medium, whereas the strongly buffered medium under the proper conditions can be depended upon to differentiate *Proteus* from all other genera in the family.

#### CONCLUSIONS

Conditions of the test, such as amount of medium, size of inoculum, diameter of the tube, and especially temperature of incubation, may markedly alter the speed of urease production by *Proteus* organisms.

The relationships of *Proteus* to other genera and species in the family *Enterobacteriaceae* with regard to urease production can be studied in weakly buffered urea medium.

In strongly buffered urea medium under the proper conditions *Proteus* can be differentiated from all other members of the family.

A rapid test to differentiate *Proteus* from other organisms in the family has been devised.

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# THE SUGAR TOLERANCE OF FOUR STRAINS OF DISTILLERS' YEAST

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In an earlier paper (Gray, 1941) it was pointed out that different strains as well as different species of yeasts vary widely in their capacities to tolerate ethyl alcohol. It was stated that a particular yeast should not be expected to produce an efficient fermentation if it was supplied with sugar in a concentration such that the alcohol produced from this sugar would be in excess of the maximum alcohol concentration which the yeast could tolerate.

The fact that a yeast exhibits a high alcohol tolerance does not mean that such a strain or species would necessarily be a more efficient organism for all industrial purposes; however, establishment of the fact that yeasts vary in their alcohol tolerances was one step in the setting up of reliable criteria by which yeasts could be selected for a particular fermentation process. The present research is another step toward establishing these criteria for different yeast strains and was projected in order to determine the relationship between initial glucose concentration of the fermentation medium and the percentage of glucose utilization for various yeasts. This problem has become increasingly important with the necessity for speeding up the production of industrial alcohol, since many plants attempt to increase their total output by increasing mash concentration, which obviously results in a decided increase in initial sugar concentration.

## EXPERIMENTAL PROCEDURE AND RESULTS

Four strains of *Saccharomyces cerevisiae* Hansen were used in these studies; they will be referred to by the numbers under which they are catalogued in the Seagram yeast stock culture collection (nos. 1, 3, 28, and 31). These yeasts were used to ferment solutions containing from 4.47 to 30.18 per cent glucose. The basal medium employed was 10 per cent yeast water (10 g of starch-free distillers' yeast extracted in 100 ml of distilled water by autoclaving for 30 minutes at 15 pounds' steam pressure and centrifuging off the suspended material; the clear liquid was then adjusted to a pH of 4.3 to 4.4). The basal medium containing glucose was divided into 25-ml lots, each of which was placed in a 50-ml Erlenmeyer flask, and sterilized by autoclaving for 20 minutes at 15 pounds' steam pressure. The initial glucose content was determined after autoclaving by the method of Stiles, Peterson, and Fred (1926). Yeast for inoculum was secured by centrifuging the cells from 24-hour-old cultures (grown in 10 per cent yeast water plus 10 per cent glucose) and resuspending in enough physiological salt solution so that the final concentration was 0.25 ml of freshly centrifuged wet yeast per ml of suspension. This results in a yeast count of 875 to 985 million cells per ml. Inoculations were made by adding 0.5 ml of such a suspension to each 25-ml portion of fermentation medium, which resulted in an

initial yeast concentration in the fermentation flasks of approximately 0.005 ml of wet yeast per ml. Inoculated flasks were then incubated under self-anaerobic conditions, without shaking, at 30 C for 72 hours, after which time final sugar analyses were made. Duplicate fermentations were run in all series. The effect of the initial sugar concentration upon the total sugar used

TABLE 1

*The effect of initial sugar concentration upon the percentage of sugar utilization by different strains of distillers' yeast*

YEAST AND SERIES NO.	AVERAGE INITIAL GLUCOSE	AVERAGE FINAL GLUCOSE	AVERAGE PERCENTAGE OF SUGAR UTILIZED	MAXIMUM THEO- RETICAL AMOUNT OF EtOH WHICH COULD BE DERIVED FROM SUGAR UTILIZED
	<i>g/100 ml</i>	<i>g/100 ml</i>		<i>per cent by weight</i>
1a	4.492	0.038	99.20	2.110
1b	9.544	0.052	99.40	4.614
1c	13.608	0.523	96.10	6.689
1d	18.160	5.852	67.75	6.282
1e	22.860	11.600	49.65	5.754
1f	26.288	17.707	32.60	4.377
3a	4.608	0.035	99.20	2.337
3b	9.404	0.054	99.40	4.778
3c	13.824	0.078	97.90	7.024
3d	18.848	1.182	93.65	9.027
3e	23.296	6.704	71.15	8.480
3f	26.624	13.367	49.70	6.774
28a	4.473	0.036	99.30	2.268
28b	8.800	0.050	99.40	4.471
28c	13.224	0.068	99.40	6.723
28d	18.320	0.276	98.45	9.073
28e	22.368	4.674	79.55	9.042
28f	27.072	11.525	57.20	7.945
31a	4.748	0.039	99.10	2.406
31b	10.568	0.054	99.40	5.372
31c	12.648	0.078	99.00	6.401
31d	14.200	0.229	99.19	7.197
31e	16.100	0.224	98.61	8.113
31f	17.930	1.255	93.00	8.521
31g	21.664	3.952	81.75	9.051
31h	26.400	11.556	56.40	7.585
31i	30.176	16.146	46.30	7.169

in 72 hours was determined by fermentations of this type, and data obtained from them are summarized in table 1 and shown in graphical form in figure 1. Duplicate fermentations were run in each instance, and the results presented are averages.

From table 1 and figure 1 it may be seen that the higher initial glucose con-

centrations resulted in a decreased percentage of sugar utilization on the part of each yeast strain, yielding curves for all yeasts that were somewhat similar. In figure 1 it will be noted that for each yeast strain the sugar utilization curve forms a straight line at the lower concentrations with a sloping off in the higher concentrations. The point at which the curve leaves the plateau may be taken as the concentration at which inhibition of sugar utilization becomes evident, and it will be noted that this point differs with the various strains.

. With the thought that perhaps the decreased sugar utilization in media of high glucose concentration resulted from alcohol inhibition, since higher alcohol concentrations would be expected as a result of the fermentation of larger amounts

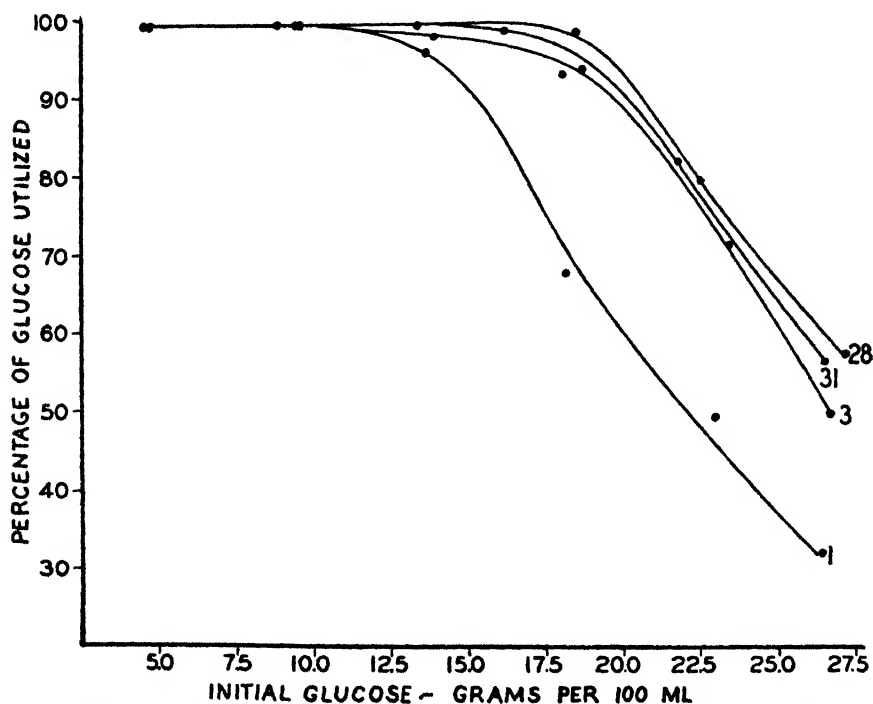


FIG. 1. THE EFFECT OF INITIAL GLUCOSE CONCENTRATION UPON PERCENTAGE OF GLUCOSE UTILIZATION BY YEASTS, NOS. 1, 3, 28, AND 31.

of glucose, calculations were made in order to determine whether it resulted from the excessive concentrations of sugar or of alcohol. The results of these calculations are plotted in curve A, figure 2. This curve was drawn by using values derived by calculating the percentage of sugar which should have been utilized at each concentration, assuming that in each instance enough sugar was utilized to produce 6.689 grams of alcohol per 100 ml (provided that the initial sugar concentration was great enough to yield this amount of alcohol). This particular alcohol concentration was selected since, in series 1c, yeast no. 1 utilized 13.085 g of glucose per 100 ml (the greatest total amount utilized in any member of the series), and the maximum theoretical resultant alcohol concentration would be

6.689 g of alcohol per 100 ml, provided there had been a 100 per cent conversion to alcohol and carbon dioxide. Examination of table 1 reveals that in series 1d, 1e, and 1f the total amount of glucose utilized was not 13.085 g per 100 ml, but 12.308, 11.260, and 8.586 g per 100 ml, respectively; in other words, above a certain glucose concentration, the higher the initial concentration of sugar in the medium, the smaller the total amount of sugar utilized in 72 hours. If alcohol were the only inhibitory factor in operation, the curve obtained by plotting the actual experimental data should coincide closely with the curve

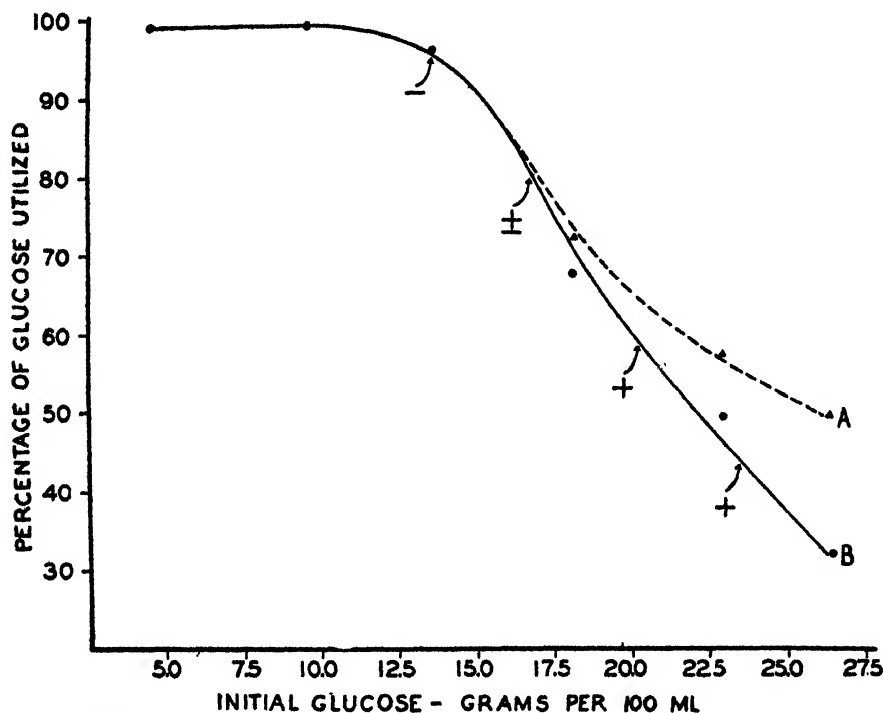


FIG. 2. THE EFFECT OF INITIAL GLUCOSE CONCENTRATION UPON PERCENTAGE OF GLUCOSE UTILIZATION BY YEAST NO. 1.

Curve A (broken) is the theoretical curve of glucose utilization based upon the assumption that the only inhibitory factor in operation is alcohol concentration; curve B (solid) is the actual utilization curve based upon data obtained from experimental fermentations. The arrows along the solid curve indicate the presence or absence of plasmolysis at the concentration designated by the arrow: -, no plasmolysis observed;  $\pm$ , partial plasmolysis; +, definite plasmolysis.

derived by such calculations as those described above. Since an examination of figure 2 reveals that this is not the case, and that in the higher sugar concentrations curve A (theoretical) lies somewhat above curve B (actual), we can assume that the inhibition of sugar utilization is not due to alcohol. It should be noted that as the sugar concentration increases, the distance between the curves also increases, which indicates an increase in inhibition with increasing sugar concentration. Obviously, curves A and B would not coincide exactly (even though alcohol were the only inhibitory factor), since 100 per cent conversion to alcohol

and CO<sub>2</sub> would not occur; however, they should approach each other more closely than they do in figure 2, and the distance between the two curves should be approximately the same at all points, if alcohol alone were the inhibitory factor.

Similar analyses and plottings of data obtained from experiments conducted with yeasts nos. 3, 28, and 31 yield sets of curves similar to those shown in figure 2, although with the different yeast strains differences between the actual and theoretical curves vary from strain to strain. From these results it would appear that the higher concentrations of glucose have an inhibitory action upon the sugar utilization of all four of the yeasts tested. Probably all yeasts exhibit this same tendency, and a survey of a large number of strains and species would undoubtedly show marked differences in their sugar tolerances.

In order to substantiate experimentally the idea derived from the results of these calculations, i.e., that the decrease in percentage of sugar utilization exhibited in table 1 was not due to alcohol concentration, the following experiment was devised: 10 per cent yeast water containing 10 per cent glucose and 6.689 g

TABLE 2

*Utilization of glucose from a medium of high initial alcohol concentration and noninhibitory glucose concentration*

Initial glucose: 10.912 g per 100 ml; initial ethyl alcohol: 6.689 g per 100 ml .

FERMENTER NO.	24 HR GLUCOSE	GLUCOSE UTILIZED	PERCENTAGE OF SUGAR UTILIZED	MAXIMUM THEORETICAL AMOUNT OF EtOH WHICH COULD BE DERIVED FROM SUGAR UTILIZED	TOTAL POSSIBLE EtOH
	<i>g/100 ml</i>	<i>g/100 ml</i>		<i>per cent by weight</i>	<i>g/100 ml</i>
1	10.104	0.808	7.44	0.413*	7.102
2	9.744	1.168	10.71	0.597*	7.286
3	9.848	1.064	9.75	0.544*	7.233
4	9.728	1.184	10.85	0.605*	7.294

\* 94 to 95% of these values would probably be more nearly correct.

of ethyl alcohol per 100 ml was prepared; four fermenters (50-ml Erlenmeyer flasks) each containing 25 ml of this medium were inoculated with yeast no. 1 by the method described in previous experiments. Sugar analyses were made at the end of 24 hours, and the results show clearly that ethyl alcohol in a concentration of 6.689 g per 100 ml will not stop glucose utilization by yeast no. 1, and, therefore, that the decreased percentages of sugar utilization exhibited by this yeast in the earlier experiments were due to a glucose, not an alcohol, inhibition. Results of this experiment are presented in table 2.

Column 6 in table 2 lists the maximum total alcohol concentration based upon the original concentration plus the maximum theoretical amount of alcohol which could be derived from the sugar utilized. Although these figures are undoubtedly slightly higher than actual, it is interesting to note their close agreement with the alcohol tolerance published for yeast no. 1 (Gray, 1941).

An examination of figure 1 reveals that the glucose utilization curves of yeasts nos. 3, 28, and 31 are very close together, and all three yeasts showed markedly



higher glucose tolerances than yeast no. 1. On the basis of their sugar tolerances we can classify the four strains as follows:  $28 > 31 > 3 > 1$ . Classifying the same yeasts on the basis of their alcohol tolerances we find that  $31 > 28 > 3 > 1$ , which would indicate that, at least for these strains, high sugar tolerance is closely correlated with high alcohol tolerance.

#### EFFECT OF HIGH GLUCOSE CONCENTRATION UPON YEAST CELL TURGOR

In an attempt to explain inhibition of sugar utilization by high glucose concentrations, consideration was given to the failure of a cell to function properly if plasmolyzed or partially plasmolyzed. For that reason, cells of yeast no. 1 were treated with glucose solutions of various concentrations in order to determine the lowest concentration which would induce plasmolysis. Slides were prepared by placing cells from a 24-hour-old yeast culture (grown in 10 per cent yeast water containing 10 per cent glucose) in a small drop of water under a cover glass. A drop of 34.37 per cent glucose solution was then placed at the edge of the cover glass, and a small piece of filter paper was placed at the opposite edge in order to pull the glucose solution under the glass by capillarity; this was repeated several times in order that the glucose solution under the cover glass might more closely approximate the stated value. It was observed that this concentration of glucose (ca. 34.37 per cent) caused immediate plasmolysis of the yeast cells. Vacuoles which are normally large in relation to total cell volume were observed to decrease in volume, and the region of the tonoplast, which in turgid cells presented a regular elliptical or circular outline, became crenate. Solutions of 26.7, 23.4, and 20.1 per cent glucose also brought about immediate plasmolysis, whereas a solution of 16.7 per cent caused only partial plasmolysis. Solutions of lower glucose concentration (13.4 and 6.7 per cent) induced no visible changes in the turgor of the cells. The solid curve in figure 2 shows that the glucose concentration at which inhibition of sugar utilization becomes evident is roughly of the same order of magnitude as that which causes plasmolysis. Yeasts nos. 3, 28, and 31 were tested in the same way, and the following results were obtained: (1) Yeast no. 3 showed definite plasmolysis at a glucose concentration of 23.6 per cent, slight plasmolysis at 18.88 per cent, and no plasmolysis at 14.16 per cent. (2) Yeast no. 28 showed plasmolysis at a glucose concentration of 18.88 per cent, very slight plasmolysis at 14.16 per cent, and no plasmolysis at 9.44 per cent. (3) Yeast no. 31 yielded results similar to those obtained with yeast no. 28, which might be expected in view of the fact that fermentation experiments demonstrated that the sugar tolerances of these two strains were about the same. The fact that different yeasts may exhibit different turgor pressures is not new, having been demonstrated by Swellengrebel (1905), who stated that a Delft yeast was plasmolyzed by glucose solutions of 0.4 to 0.5 molar concentration (7.2 to 9.0 per cent) and that a wine yeast was plasmolyzed by 1.2 molar glucose (21.6 per cent). This investigator did not attempt to correlate plasmolysis with sugar utilization, however.

From these observations we may infer that the sugar inhibitions exhibited are due at least in part to osmotic phenomena, and that if a yeast cell is placed in a

sugar solution of higher osmotic value than that of the vacuole contents, the cell would be at least partially plasmolyzed and undoubtedly would be unable to function in a normal way. Microscopic examination should serve to give a rough index to the sugar tolerance of a yeast. However, too much dependence should not be placed on results so obtained, since it is difficult to detect the exact onset of plasmolysis with the rather crude procedure employed, which fact may partially explain the discrepancies that appear between the results obtained by microscopic examination and those obtained from actual measurements of sugar utilization. One could, by using proper stains and by accurately measuring vacuole diameters, arrive at a more nearly exact value for plasmolyzing concentrations. In the final analysis, however, the fermentation method seems to be more suitable for the accurate determination of sugar tolerances.

One should not overlook the possibility that the inhibition of sugar utilization exhibited in these studies is due largely to an initial inhibitory effect on the yeast cell, and that if the fermentations herein described had been allowed to proceed for periods longer than 72 hours, the sugar utilization curves would be different. With regard to this possibility, however, it should be stated that an alcoholic fermentation of greater than 72 hours' duration would not be of much value from the industrial point of view.

There is a further possibility that inhibition of sugar utilization by glucose in high concentrations is not due entirely to osmotic phenomena (Geiger-Huber, 1935). This investigator studied the effects of varying glucose concentration on the respiratory rates of four yeasts, "beer yeast," "bakers' yeast," *Debaryomyces tyrocola*, and an "air yeast," and found that the optimum concentration for maximum respiration varied with the four yeasts. These values ranged from  $m/30$  for the air yeast to  $m/225$  for bakers' yeasts. Since the most concentrated glucose solution employed by Geiger-Huber in his studies was  $m/6.25$  (2.88 per cent), it is doubtful that any plasmolysis occurred. Also, since in the range of sugar concentrations from  $m/6.25$  to  $m/10,000$  optimal concentrations for maximum respiration were exhibited by each yeast, it seems possible that sugars may exert certain effects other than osmotic ones on the yeast cell. This problem will bear further investigation.

#### SUMMARY

Four strains of *Saccharomyces cerevisiae* Hansen have been investigated, and it has been shown that the ability of these yeasts to utilize glucose may be affected by the initial glucose concentration of the medium to be fermented, and also that different yeasts vary in their abilities to tolerate high sugar concentrations.

In order to obtain a possible explanation of the inhibition of sugar utilization demonstrated in fermentation experiments, the various yeast strains were treated with glucose solutions of different concentration in order to determine the concentrations necessary to induce easily visible plasmolysis. The results of these experiments indicate that the inhibition at high glucose concentrations is due, at least in part, to osmotic phenomena.

It is suggested that a rough determination of the sugar tolerance of a yeast

can be made simply by the use of the microscope and glucose solutions of various strengths. However, for accurate determinations of sugar tolerance the fermentation method is recommended.

#### ACKNOWLEDGMENTS

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# OXIDATION-REDUCTION POTENTIALS OF THE CONTENTS OF THE GASTROINTESTINAL TRACT

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Little work has been reported on oxidation-reduction potentials in the gastrointestinal tracts of men or animals, in spite of the fact that the intestines are known to be the site of powerful reduction processes associated particularly with bacterial actions occurring therein. These oxidation-reduction potentials may be expected to be of influence on some of the numerous chemical reactions taking place in the gastrointestinal tract. Besides being a reflection of the intensity and character of bacterial growth, the potentials may influence the nature and extent of such flora.

Bergeim (1924) reported observations on the reduction of hydrated ferric oxide in the gastrointestinal tract under various conditions of diet, giving a semiquantitative measurement of reduction intensities. Actual measurements of oxidation-reduction potentials were not however carried out.

The present paper gives the results of some observations on the oxidation-reduction potentials in different parts of the gastrointestinal tract of animals as influenced by diet and by certain sulfonamides.

It is, of course, realized that the potential measurements do not tell us anything as to the amount or exact nature of the reducing substances present and are in this sense preliminary in nature. They do however serve to define the usual limits of reduction intensity in the gastrointestinal contents and to indicate whether or not very small amounts at least of a given substance are likely to be reduced in a certain section of the tract.

## EXPERIMENTAL

Albino rats, about 150 g in weight, were fed various diets for periods of from a few days to several weeks. The animals were then killed and material collected from the stomach, the upper half of the small intestine, the lower half of the small intestine, from the cecum, and from the colon. Oxidation-reduction potentials were determined on these materials by using a bright platinum electrode (Beckmann no. 1281 with 5-mm-square platinum foil checked at intervals against a standard quinhydrone solution) and a potentiometer (Leeds and Northrup 4661-A1). Determinations were begun immediately after death, starting with the contents of the stomach and following with those of the small and then of the large intestine. Material was left in the organ until just before the estimation was to be started. The stomach or intestine was then cut open and the contents carefully squeezed out. Determinations were made in open dishes exposed to air and at room temperature. The room temperature varied from 25 to 28 C. No temperature correction was made since such correction would have been with-

in the limits of error of the general procedure. Nor was an attempt made to correct to body temperature for the same reason, and also because such a correction applied to the varying material would have been an approximation. The material was mixed by gently stirring. No water was added. The platinum foil was entirely covered by the mixture, with which the calomel electrode was also put in contact. The electrode was moved about at intervals in the material to avoid the measurement of merely local potentials. Little difficulty was met with on this score. None of the materials studied were so solid that this could not readily be done. Readings were taken as soon as equilibrium was reached. Equilibrium was regarded as the time when no further change in reading was found on 3 to 4 minutes' longer standing. This period usually occurred after 15 to 20 minutes, with a shorter time for the acid gastric contents. In nearly all cases the readings became gradually more negative, and the most negative reading was taken. In a few instances, on the raw apple diet only, did readings gradually become more negative and then turn in a positive direction indicating oxidation by contact with the air. Here the most negative value obtained was recorded. It is possible that the oxidase of the apple was responsible for this behavior. With the other diets this did not occur even in certain cases where contact with the air was continued for an hour or longer. Apparently oxygen did not readily penetrate the materials, perhaps being used up at the surface. The bacterial action influencing the potential continues, of course, during the determination. At the beginning it was thought possible that an atmosphere of nitrogen might be necessary for the carrying out of the determinations using electrodes introduced directly into the gut. In certain instances such precautions may be desirable, but in the present investigation we found no reason to think them necessary to obtain results we believe fairly representative of the situation existing *in vivo*. In several instances the electrodes were introduced into the cecum of an animal *in situ* through small incisions in the wall, the animal being kept in a glass chamber with a stream of nitrogen gas being passed over the lower part of the body. The results were essentially the same as those obtained on the same material measured in an open dish. The measurements reported were, however, made on materials removed from the body.

A variety of diets were used. The meat was an air-dried powdered beef. The bread was an average commercial fortified white bread dried at 40 C and ground to a coarse powder. A basal diet used was 75 per cent bread and 25 per cent meat. The diets were given *ad libitum* for periods of from 7 to 36 days. Carbohydrates were added to this basal diet in the proportion of 100 parts of carbohydrates to 150 of basal diet. The starch used was corn starch. The average results given are for three animals in a group. No wide variations among the individual animals in the groups were observed. Nor did the time the rats were on this diet in general have much effect after the first week, except in the case of the bread-meat diet with added lactose.

Results are presented in table 1. Oxidation-reduction potentials of original foods mixed with twice their weight of distilled water (pH 5) are also included.

Potentials are expressed as millivolts. The sign is in accord with the European

convention commonly used by bacteriologists and biochemists and is referred to the standard hydrogen electrode.

TABLE 1

*Oxidation-reduction potentials of the contents of the gastrointestinal tracts of rats on different diets*

DIET COMPOSITION	E <sub>h</sub>	pH	STOMACH		SMALL INTESTINE				CECUM		COLON	
			E <sub>h</sub>	pH	Upper		Lower		E <sub>h</sub>	pH	E <sub>h</sub>	pH
					E <sub>h</sub>	pH	E <sub>h</sub>	pH				
I. Bread.....	+350	4.6	+220	4.7	-98	6.4	-89	6.8	-185	6.1	-120	6.4
II. Meat-bread....	+216	4.7	+138	4.3	-67	6.2	-90	6.9	-202	5.8	-103	6.3
III. Meat.....	+184	4.7	+91	4.7	-64	6.4	-83	6.6	-237	6.9	-168	7.1
IV. Meat-bread + starch....	+226	4.9	+149	4.5	-56	6.0	-149	6.6	-202	6.8	-107	6.2
V. Meat-bread + sucrose...	+228	4.9	+150	4.2	-90	6.2	-100	6.4	-122	6.4	-26	6.0
VI. Meat-bread + dextrin...	+238	4.9	+96	3.9	-154	6.3	-127	6.4	-150	5.2	+36	5.8
VII. Meat-bread + glucose...	+210	4.9	+53	4.8	-179	6.1	-235	6.5	-209	5.9	-167	6.1
VIII. Meat-bread + lactose after 13 days.....	+224	4.9	+154	6.1	+93	6.3	+104	6.5	+164	4.5	+128	4.8
After 20 days.....	+224	4.9	+158	3.5	-62	5.6	-214	6.4	+28	5.2	+78	5.5
After 36 days.....	+224	4.9	+114	5.3	-96	6.4	-59	6.1	-64	5.2	-100	5.6
IX. Whole milk powder.....	+316	6.8	+65	5.3	-194	6.3	-179	6.3	-206	6.1	-174	6.3
X. Milk powder + lactose 1:1.....	+300	6.8	+57	3.8	-139	6.1	-63	6.4	-88	5.6	-80	5.5
XI. Soft-curd milk powder.....	+212	4.7	+175	4.3	-182	6.1	-88	6.2	-183	5.6	-139	6.8
XII. Raw apple....	+560	3.5	+499	3.2	+114	5.4	+68	7.3	-130	7.2	+136	7.4
XIII. Meat-bread + sulfasuxi- dine 1%.....	+214	4.9	+148	3.9	-154	6.5	-135	6.5	-252	5.9	-133	6.4
XIV. Meat-bread + sulfa- guanidine 1%.....	+220	5.0			-93	6.8	-158	6.9	-266	6.4	-164	6.5

## DISCUSSION

The diets used gave potentials of about +200. Reducing constituents of the diets (reducing sugars, etc.) could not therefore be responsible for the negative values generally found in the intestines. Such negative values must therefore be due primarily to products of bacterial action. In the stomach only positive

values were obtained, indicating in general a very limited bacterial action. The pH values in the stomach were usually such as would tend to keep down bacterial growth, but some bacteria usually appeared to be present in the rat's stomach. As might be expected, the most negative values were usually found in the cecum where bacterial action is usually most intense. Values around  $-200$  were frequently found.

More surprising was the fact that reduction intensities of  $-100$  or greater were not uncommon in either the upper or lower half of the small intestine. We did not make bacterial counts, but Porter and Rettger (1940) studied rats on a variety of diets and found bacteria consistently in relatively small but appreciable numbers in the stomach, duodenum, and jejunum, with increasing numbers in the upper ileum and large numbers in the lower ileum and cecum. Except on meat diets, *Lactobacillus acidophilus* types of organisms predominated. The number of coliform type organisms was low in the upper part of the tract but greatly increased in the lower ileum and cecum. They also found the flora, though capable of change by diet, to be fairly stable under rather wide variations of diet.

Our results indicate that the oxidation-reduction potentials in the contents of the gastrointestinal tract of the rat are fairly stable through considerable variations of diet. Thus meat and bread diets gave rather similar results. The addition to bread-meat diets of large amounts (40 per cent) of starch, glucose, sucrose, or dextrin did not produce great changes. Lactose, however, had a much greater effect. Positive readings were obtained throughout the tract even after 13 days on this diet. After 36 days the values had shifted to the negative side, but reduction intensities were still definitely lower than on other diets. The shift was accompanied by a decrease in acidity of the cecal contents and probably by an increase in coliform bacteria as compared with the predominating aciduric organisms, since we have noted such changes in other animals on this diet. The results showing the much greater effect of lactose than of other carbohydrates in decreasing the reduction intensities in the tract are in agreement with the observations of Bergeim on iron reduction. The fact that this high lactose diet was the only diet giving rise to oxidation-reduction potentials deviating much from average findings, and that even here there was a gradual return toward this average, emphasizes the relatively constant findings even with widely varying diets. This is presumably in large part due to the tendency for a relatively constant flora to be maintained in the tract. Since rather similar findings were obtained with meat and with bread diets, although the aciduric flora must have been greatly less on the meat diet as compared with coliform organisms, it seems clear also that some of the constancy of results is due to the fact that both types of organisms produce strongly reducing products. It must also be constantly borne in mind that the potentials measured do not indicate the quantity of reducing substances present. Hence a small number of bacteria may give rise to potentials approaching those given by a much larger number, since even a relatively small amount of a strongly reducing bacterial product may give rise to fairly strong negative potential

values. In this sense of measuring the quantity of reducing substances formed, a procedure of the type of the iron reduction method (where an excess of reducible substance is maintained) would give more significant data, as would also oxidation-reduction titrations of the intestinal contents. The latter would also be essential in any study of the components of the oxidation-reduction systems present. The purpose of the present investigation was, however, quite different, i.e., to establish the general limits of oxidation-reduction potentials in the gastrointestinal tract under different conditions of diet for their general bacteriological interest. It was also desired to obtain data for predicting what substance might be expected not to be reduced in the intestines, as well as what substances might be expected to be reduced there if present only in very small amounts. Naturally, in the study of the reduction of a particular substance in the tract it may be desirable to make a quantitative study.

The results with raw apple are undoubtedly influenced by the high positive value (+546) given by the apple itself, for which the acidity of the apple and its oxidase content are probably responsible. The pH values must of course be considered in connection with all of the findings given. It will be noted however that with most diets no great variations of intestinal pH are observed.

Since sulfasuxidine and sulfaguanidine have been commonly employed as intestinal antibacterial agents, some alteration in potentials by these agents might have been expected. No such differences were, however, observed. This is apparently due to the fact, as shown by Light *et al.* (1942) and by Gant (1944), that whereas these drugs reduced for a time the number of coliform organisms in the intestines, there was a corresponding increase in the number of enterococci and other bacteria, so that total bacterial counts remained about the same. Since both types of organisms have strong reducing properties, a change in potentials in the intestines would therefore not necessarily be expected.

Observations on the application of these findings to studies of the reduction of specific chemical substances in the intestines are being continued. Much more work is required to determine the exact bacteriological and chemical significance of such findings.

#### SUMMARY AND CONCLUSIONS

Determinations were made of oxidation-reduction potentials (at different levels) of the contents of the gastrointestinal tract of rats on a variety of diets. The  $E_h$  values of the diets studied averaged about +200. Average values for the stomach contents were about +150, for the contents of the upper small intestine around -100, for the lower small intestine about the same, and for the cecum around -200. This is in line with the increasing number of bacteria as food passes downward. Widely varying diets, such as milk, meat, and bread diets, gave rather similar results. High lactose diets were most effective in decreasing reducing intensity. Other carbohydrates had much less effect. Sulfasuxidine and sulfaguanidine had little effect on potentials. The results are believed to be due in part to the fact that the flora tends to remain constant and also that a shift in the ratio of aciduric to coliform types of organisms may



not greatly affect the potentials since both have strongly reducing effects. The results may serve as a guide to more detailed studies of the reduction of various chemical substances in the gastrointestinal tract, and the relation thereto of the bacterial flora.

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# A PROPOSED BIOCHEMICAL BASIS FOR THE GENUS *PSEUDOMONAS*

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In a recent note on the transfer of red halophilic bacteria from the genus *Serratia* to the genus *Pseudomonas* on the basis of their marine origin, their flagellar arrangement, and their physiology, Lochhead (1943) states, "The inclusion of red, pigmented, halophilic bacteria in the genus *Pseudomonas* naturally requires a corresponding widening of the generic description."

In the current edition of *Bergey's Manual* (1939), the genus *Pseudomonas* comprises "soil and water bacteria, usually producing a water-soluble pigment which diffuses through the medium as a bluish-green or yellowish-green pigment." The enlargement of this genus as suggested by Lochhead is undoubtedly desirable, and the time is perhaps at hand when the genus *Pseudomonas* can be put on a biochemical basis (at least tentatively) to include a large number of other chromogenic bacteria at present classified in other genera on the basis of pigment color. To continue to classify bacteria mainly by the color of their pigments, without regard to their known or probable chemical constitution, seems as irrational as it would be for a chemist to classify his reagents on the basis of color alone.

The suggested biochemical basis is that members of the genus *Pseudomonas* should comprise those rod-shaped bacteria which produce either pigments which are phenazine derivatives, regardless of their color, or water-soluble fluorescent pigments, or both. This would require a certain amount of chemical work before a given species is *definitively* assigned to the genus, but the work would not be unduly onerous since the phenazine pigments (at least those already known) can usually be isolated in pure form without undue difficulty, and the presence of a phenazine nucleus in the molecule demonstrated by destructive distillation with zinc dust or by other methods of strong reduction. The ultraviolet absorption spectra of the phenazine pigments are also characteristic. The water-soluble fluorescent pigments are readily demonstrated by inspection of the culture concerned. As a tentative basis, rod-shaped bacteria might be classed in *Pseudomonas* if they produce water-soluble pigments of any color, particularly if the pigments vary in color under different conditions of pH and of oxidation and reduction, and can be extracted from alkaline solution with chloroform.

Such an approach would lead to a much more rational classification from a physiological point of view, since the phenazine pigments (and possibly the fluorescent pigments) apparently play a significant part in the life of the bacteria which secrete them, although their exact role is not yet entirely clear. According to Kramer (1935), McIlwain (1941), Schoental (1941), Singh (1942), and others, the phenazine pigments are of possible functional value in antagonizing the growth of competing organisms. According to Stheeman (1927),

Elema (1931), Friedheim (1931, 1932), Reed and Boyd (1933), and others, the phenazine pigments are apparently involved in the oxidation and reduction processes of the bacteria which produce them.

Two pigments of species at present classed as *Pseudomonas* have been chemically investigated in detail and have proved to be phenazine derivatives. The pigment pyocyanine from *Pseudomonas aeruginosa* (*Bacillus pyocyaneus*) is a quinoid complex consisting of two oxidized molecules of 1-hydroxy, 5-hydro, 10-methyl phenazine (Wrede and Strack, 1929), whereas chlororaphine from *Pseudomonas chlororaphis* (*Bacillus chlororaphis*) is a quinhydrone-like complex of phenazine-1-carboxamide and dihydrophenazine-1-carboxamide (Kögl and Postowsky, 1930). In addition, the violet, water-soluble pigment of the so-called *Chromobacterium iodinum* described by Davis (1939) was demonstrated to be 1,2-dihydroxyphenazine-9,10-di-*N*-oxide by Clemo and McIlwain (1938). Since the pigment is distinctly different from violacein, the water-insoluble pigment of *Chromobacterium violaceum* (*Bacillus violaceus*), which appears to be a pyrrole or indigo derivative of high molecular weight (Tobie, 1938), we have already suggested that *C. iodinum* be reassigned to the genus *Pseudomonas* under the name of *Pseudomonas clemoi* or *P. iodina* (Tobie, 1939).

In addition to the bacteria at present classed in *Pseudomonas*, there is a large number of chromogenic bacteria having pigments whose properties strongly suggest that they are or may be phenazine derivatives. Among these are *Bacillus polychromogenes* (Chamot and Thiry, 1900), *Bacterium* (*Chromobacterium*) *viscofucatum* (Harrison and Barlow, 1905), *Bacterium coelicolor* (Müller, 1908), *Flavobacterium lasseuri* (*Bacillus le monnieri*, Lasseur, 1913), *Serratia anolium* (Duran-Reynals and Clausen, 1937), and many others. As rapidly as chemical work demonstrates a phenazine nucleus in the pigment molecule, such organisms should be reassigned to the genus *Pseudomonas*.

On the other hand, the proposed reclassification would exclude certain bacteria from the genus *Pseudomonas*. Among these is the so-called *Pseudomonas beijerinckii*, the insoluble purple pigment of which appears to be the calcium or magnesium salt of tetrahydroxyquinone (Kluyver *et al.*, 1939). Another is possibly *Bacillus indigoferus* Voges, studied under the name of *Pseudomonas indigofera* by Elazari-Volcani (1939), the water-insoluble, blue pigment of which was found to differ considerably from indigo. Possibly this organism should be retained under the genus *Chromobacterium* in which it is at present tentatively classified in the Bergey system (1939). Whenever an organism classed as a *Pseudomonas* is found to have a nonphenazine or nonfluorescent pigment, it should be placed in a different or even in a new genus. The generic designations *Quinobacterium* or *Phenobacterium* might be used for bacteria the pigments of which are quinoid or phenolic respectively.

Organisms of the *Pseudomonas fluorescens* group (producing water-soluble, greenish, fluorescent pigments) are retained under the genus *Pseudomonas* in the proposed reclassification, since they are very closely related in cultural characteristics to the organisms producing phenazine pigments. The relationship (if any) between the fluorescent pigments and the phenazine pigments is

still obscure. According to Turfreijs *et al.* (1938), the green fluorescent pigment of *P. fluorescens* has the apparent formula  $C_{32}H_{41}O_8N_7$ , although the nucleus of the molecule was not determined. Turfitt (1936, 1937) presented evidence that the fluorescent pigments of *P. aeruginosa*, *P. fluorescens* var. *liquefaciens*, and *P. fluorescens* var. *nonliquefaciens* may be identical in chemical composition. Many organisms of the present genus *Phytomonas* (plant pathogens) of the Bergey system closely resemble the *Pseudomonas fluorescens* group on the production of a green, water-soluble, fluorescent pigment. Since pathogenicity is not a very satisfactory taxonomic criterion, it is suggested that such fluorescent plant pathogens might well be included in the genus *Pseudomonas*.

#### SUMMARY

It is suggested that the genus *Pseudomonas* should comprise those rod-shaped bacteria which produce water-soluble phenazine pigments, regardless of the color of the pigments, or water-soluble, fluorescent pigments, or both. Since these pigments apparently have a significant rôle in the life of the bacteria which secrete them, the proposed classification would be much more rational from a biochemical and physiological point of view. Bacteria producing pigments of a demonstrably different chemical nature should be excluded from the genus *Pseudomonas*.

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# A MEDIUM CONTAINING AN ACID CASEIN HYDROLYZATE FOR USE IN TESTING DISINFECTANTS

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After the introduction of phenols of high germicidal potency as disinfectants, various laboratories began to experience discrepancies in results obtained by the F. D. A. phenol coefficient procedure. These variations were particularly marked when the test organism, *Staphylococcus aureus*, was used. The increased demand for disinfectants and antiseptics for war use and the increased emphasis on *S. aureus* as a test organism have caused these variations to become more evident and important.

The U. S. Food and Drug Administration (1931) established an official procedure for the phenol coefficient test. Liebig's beef extract and Armour's special peptone were specifically designated as components of the culture medium for the test organisms, *Eberthella typhosa* and *S. aureus*. At the present time, Liebig's beef extract is practically unobtainable.

Wright (1917) and Reddish and Burlingame (1938) have shown that different brands of peptone used in the culture medium may cause differences in coefficient values. Brewer (1943) demonstrated that different lots of the same brand of peptone caused as much as 100 per cent variation in phenol coefficient values.

The English workers, Gladstone (1937), Knight (1937a, 1937b), Richardson (1936), and Fildes *et al.* (1936) have proved that *S. aureus* can grow in a synthetic medium in which amino acids furnish the sole source of nitrogen with the exception of that contained in the added growth factors. However, the use of the amino acids as a source of nitrogen is rather expensive, and, in some cases, their sterilization is laborious.

Woolley and White (1943), in a study of microbial inhibition of several organisms including *S. aureus*, used an acid hydrolyzate of casein ("casamino acids") as the main source of nitrogen for bacterial growth. The complete medium is as follows: glucose, 0.25 per cent;  $K_2HPO_4$ , 0.50 per cent; NaCl, 0.25 per cent; casamino acids (Difco), 0.25 per cent; *l*(-)-tryptophane, 25 mg per L; uracil, 5 mg per L; niacin, 1 mg per L; thiamine, 0.01 mg per L; biotin, 0.001 mg per L; and 2.5 ml per L of salt solution (Snell and Strong, 1939).

As this medium seemed to have promise of being more nearly reproducible than the F. D. A. peptone medium, it was used as a starting point in this study. The various components of the mixture were studied in order to find which were

<sup>1</sup> Mr. H. G. Dunham of the Difco Laboratories, Detroit, Michigan, kindly supplied the "casamino acids" for this study. A chemical analysis furnished to us by the Difco Laboratories is as follows: ash 42.4 per cent, NaCl 39.0 per cent, total nitrogen 8.0 per cent, amino nitrogen 6.0 per cent.

essential for supporting growth and maintaining proper resistance of the test organisms to phenol.

#### PROCEDURE

*S. aureus* was used in preference to *E. typhosa* in establishing the test medium, as the former organism is more exacting in its requirements for growth.

Smyth (1934) has observed the variations in resistance of *S. aureus* to phenol caused by such factors as differences in age of culture, temperature of incubation, and size of inoculum. Care was taken, therefore, to control these factors as closely as possible. A mercury thermoregulator was used in controlling the temperature of incubation between 38.1 and 38.3 C. Transfers were made at the same time each day, followed by determinations of resistance to phenol of the 24-hour cultures. Resistances were run daily, except Sunday.

One culture of *S. aureus*, no. 209, was obtained directly from the F. D. A. collection, and eight substrains of this culture were furnished by laboratories actively interested in the testing of germicides. Three substrains, arbitrarily identified as nos. 7, 8, and 9, were used in preliminary studies, since these cultures were available at the time the work was started.

#### THE EFFECT OF VARIOUS COMPONENTS OF THE MEDIUM ON RESISTANCE TO PHENOL

**Vitamins.** The English school of workers found early in their study of the nutrition of *S. aureus* that a supplement containing unidentified vitamins was needed in addition to the known components of the medium. Knight (1937a, 1937b) first resolved the supplement and found that thiamine, or its pyrimidine and thiazole components, along with niacin could substitute for the vitamin complex which had previously been used.

In view of this work, only thiamine hydrochloride and niacin were included in our test mediums. However, the concentration of thiamine was increased from 0.01 mg per L to 1 mg per L, the concentration suggested by Brewer (1942). The latter author reported that an increased concentration of thiamine helped to increase the resistance of *S. aureus*.

Although Porter and Pelczar (1940) reported that certain strains of *S. aureus* required biotin to initiate growth, and that growth already initiated was more luxuriant in the presence of increased amounts of biotin within certain ranges, the F. D. A. no. 209 strain did not require added biotin under the conditions of our test.

**Glucose.** Since Woolley and White's (1943) medium, as well as the synthetic medium developed by the English workers, contained glucose, the question arose whether this compound would be required under the conditions of our test. Preliminary study indicated that preformed glucose was not required and, therefore, was not included in later experiments.

**Inorganic salts.** Snell and Strong's (1939) salt solution contains  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , and  $\text{NaCl}$ . The  $\text{NaCl}$  of this solution was omitted, since this quantity is insignificant in comparison with that supplied by the casein hydrolyzate.

*pH of the medium.* The F. D. A. procedure calls for a medium in the pH range of 6.8 to 7.2, as this is probably within the optimum range for growth (Zinsser and Bayne-Jones, 1937). The results of table 1 indicate that the resistance of substrains 7 and 9 grown at pH 6.5 is distinctly lower, whereas that of substrain 8 is somewhat less than that obtained with the same organism grown at pH 7.5. All subsequent mediums were adjusted to a pH range of 7.2 to 7.4.

*l(-)Tryptophane.* Although Fildes and Knight (1933) found that *S. aureus* required a tryptophane supplement when cultured in a gelatin hydrolyzate, Gladstone (1937) reported that 21 out of 25 strains of *S. aureus* were able to grow in an amino acid medium devoid of tryptophane. Gladstone did not include strain 209 in his tests.

TABLE 1  
*Effect of pH on resistance to phenol of Staphylococcus aureus, no. 209*

SUBSTRAIN	pH	FREQUENCY OF RESISTANCE TO PHENOL					
		1:60 ++-	1:60 +--	1:65 ++-	1:65 +--	1:70 ++-	1:70 +--
7	6.5				1	14	7
8	6.5	2	5	6	5	1	
9	6.5				5	14	1
7	7.5			14	4		
8	7.5	5	5	6	1	1	
9	7.5		6	8	3	1	

Frequency of resistance to phenol dilutions in this and subsequent tables represents the number of times that the indicated resistance was obtained during the course of the experiment.

+ indicates growth, - indicates no growth, after exposure time intervals of 5, 10, and 15 minutes in the respective sequence of the signs. Incubation for growth, 48 hours at 37 C.

Because of the limited supply of casein hydrolyzates, subcultures from all test solutions were made in F.D.A. medium.

Three substrains of *S. aureus* 209 were cultured in two mediums which were identical except for their tryptophane content. In the one case *l(-)*tryptophane was added to the medium. The casein hydrolyzate did not contain detectable amounts of tryptophane according to information furnished by the manufacturer (Dunham, 1944).

Table 2 shows that tryptophane is not required for the proper maintenance of resistance to phenol. In fact, the substrains tested seem to be more resistant in the absence of tryptophane. Hence, the F. D. A. strain of *S. aureus* would appear to belong to the group of staphylococci which does not require preformed tryptophane. At least, the required concentration is less than that detected by chemical analysis.

*Uracil requirement.* Richardson (1936) reported that *S. aureus* required uracil when grown anaerobically upon a mixed amino acid medium containing the other required accessory growth factors. However, he found that cultures



of both *E. typhosa* and *S. aureus* synthesized uracil when grown under aerobic conditions. It was, therefore, of interest to see whether uracil was required for growth and resistance under the conditions of our test. Table 3 presents experiments in which the effect of uracil was studied. A comparison of each of the three substrains grown in the two mediums indicates that although growth takes place in the absence of uracil, its presence improves the resistance of the organisms.

In view of these findings, uracil has been included in subsequent studies.

TABLE 2

*Effect of l(-)tryptophane on resistance to phenol of Staphylococcus aureus, no. 209*

SUBSTRAIN	l(-)TRYPTOPHANE	FREQUENCY OF RESISTANCE TO PHENOL					
		1:60 ++-	1:60 +--	1:65 ++-	1:65 +--	1:70 ++-	1:70 +--
7	25 mg	2	11	1		4	16
8	25 mg	6	20	6	3		
9	25 mg	3	10		16	2	1
7	No added tryptophane	3	8	3	12	12	10
8	No added tryptophane	23	86	9	13		
9	No added tryptophane	1	12	7	9	4	

TABLE 3

*Effect of uracil on resistance to phenol of Staphylococcus aureus, no. 209*

SUBSTRAIN		FREQUENCY OF RESISTANCE TO PHENOL				
		1:60 ++-	1:60 +--	1:65 ++-	1:65 +--	1:70 ++-
7	Uracil 5 mg/L	3	8	1	5	
8	Uracil 5 mg/L	23	86	9	13	
9	Uracil 5 mg/L	1	12	1	3	
7	No added uracil			2	14	4
8	No added uracil	1		4	8	1
9	No added uracil	2	6	8	1	

*Effect of casein hydrolyzate concentration.* Culture 8 was chosen for evaluation of the amount of casein hydrolyzate to be used because it would decrease the labor of testing and because this substrain had been rather consistent in its resistance to phenol in previous experiments.

The results, as illustrated in table 4, indicate that a concentration of 0.5 per cent of the casein hydrolyzate is optimum for obtaining the proper resistance to phenol. At a lower concentration (0.25 per cent) the resistance to phenol was lowered (lot S14310 is an exception in that there is little difference), whereas at a higher concentration (1 per cent) the resistance of the cultures is similar to that obtained in the presence of 0.5 per cent casein hydrolyzate.

TABLE 4

*Effect of concentration of casein hydrolyzate of Staphylococcus aureus, no. 209, substrain 8*

CONCENTRATION OF CASEIN HYDROLYZATE	LOT NO.	FREQUENCY OF RESISTANCE TO PHENOL				
		1:55 +--	1:60 ++-	1:60 +--	1:65 ++-	1:65 +--
<i>per cent</i>						
0.25	S14310		23	86	10	18
0.50	S14310		22	67	2	16
0.38	46127*		2	14	2	3
0.76	46127	10	8	20	1	
1.00	46127		7	14		
0.25	46513			4	4	6
0.50	46513		14	51	3	5
1.00	46513		4	8	4	2
0.50	46809	23	6	26	7	
1.00	46809	2	2	11	2	1

\* 0.38% of this technical lot is equivalent in nitrogen to 0.25% of the purified lots.

TABLE 5

*Effect of various lots of casein hydrolyzate on resistance to phenol of Staphylococcus aureus, no. 209*

SUB- STRAIN	CASEIN HYDROLYZATE LOT NO.	FREQUENCY OF RESISTANCE TO PHENOL				
		1:55 +--	1:60 ++-	1:60 +--	1:65 ++-	1:65 +--
8	S14310		22	67	2	16
8	46127 (tech)	10	8	20	1	
8	46513		14	51	3	5
8	46809	23	6	26	7	
8	1.0% Peptone* (F. D. A. Control)		10	37	10	17

\* Armour's peptone, lot 2005B.

The results of these studies justified the use of the following formula in determining the reproducibility of various lots of the casein hydrolyzate:

$K_2HPO_4 \cdot 3H_2O$ ..... 3.0 g  
 Casamino acids..... 5.0 g or N equivalent in technical lots  
 Salt solution\*..... 2.5 ml  
 Uracil..... 5.0 mg  
 Thiamine hydrochloride..... 1.0 mg  
 Niacinamide..... 1.0 mg  
 $H_2O$ ..... 1 L  
 pH adjusted to 7.2 -7.4

\*  $\left\{ \begin{array}{l} 10 \text{ g } MgSO_4 \cdot 7H_2O \\ 0.5 \text{ g } FeSO_4 \cdot 7H_2O \\ 0.5 \text{ g } MnSO_4 \cdot 4H_2O \end{array} \right\}$  per 250-ml solution

Table 5 includes the resistance obtained with four lots of hydrolyzate along with F. D. A. medium as a control. The results illustrated in this table indicate that the various lots of casein hydrolyzate produced cultures with resistance to phenol as great or greater than that of organisms cultured in F. D. A. medium.

The differences in resistance to phenol caused by the four lots of hydrolyzate are noticeable, cultures grown in lots 46127 and 46809 being the most resistant. However, this difference in resistance is not marked, particularly when one considers the variation obtained in the case of any one medium alone.

At the time of these experiments lot 2005B was the only official test lot of peptone available. Although organisms cultured in this particular peptone had a resistance similar to cultures grown in the casein hydrolyzate, it has been

TABLE 6

*Resistance of substrains of Staphylococcus aureus, no. 209 grown, in the same medium*

SUBSTRAIN		FREQUENCY OF RESISTANCE TO PHENOL					
		1:55 +---	1:60 ++-	1:60 +---	1:65 ++-	1:65 +---	1:70 ++-
7	0.76% casein hydrolyzate lot 46127		3	15		3	
8		10	8	20	1		
9		11	3	9			
10				1	5	19	
12			1	5	6	15	
13		14		4		6	
14			5	23			
15			3	10	3	7	
8	F.D.A. medium (control) 1.0% Armour's peptone lot 2005B		10	37	10	17	
10			1	13	1		
11					1	4	6
12				9	1	1	
13			11	2			
14					9	5	
15			1	12			

our experience in the past to have official lots of peptone which produced cultures that were less resistant to phenol. Often it has been difficult to obtain a culture which would resist phenol at a dilution of 1:70 for more than 5 minutes.

Substrain 8 has been cultured in the medium containing casein hydrolyzate, lot S14310, for a period of nearly eight months and has continued to give a satisfactory standard of resistance to phenol.

Continued transfer in the new medium tends to produce cultures which are not so granular as those frequently experienced with the F. D. A. medium.

#### VARIATION OF SUBSTRAINS OF *S. AUREUS*, F. D. A. NO. 209

It soon became evident in the course of this study that the variations between substrains of *S. aureus* would have to be taken into account. Several substrains were cultured in the same medium under identical conditions, and determinations of resistance of 24-hour cultures to phenol were made (table 6). Resistances

obtained in the case of the F. D. A. medium are included in order to demonstrate that the substrain variation is not peculiar to the medium containing the hydrolyzate.

These results show that the substrains vary considerably in their resistance to phenol regardless of the medium in which they are grown. Certainly the need for closer control of the test organism as well as the need for a reproducible medium is emphasized.

TABLE 7

*Phenol coefficients versus Staphylococcus aureus, no. 209 substrain 8, cultured in F. D. A. and experimental medium*

F. D. A. MEDIUM		CASEIN HYDROLYZATE	
Date detmn.	Phenol coef.	Date detmn.	Phenol coef.
Orthophenylphenol			
4/21/44	21		
4/22/44	19		
5/ 6/44	20	5/ 6/44	20
5/ 8/44	23		
5/11/44	22	5/11/44	22
		8/ 7/44	20
Chlorinated orthophenylphenol			
4/20/44	110		
4/22/44	115		
		5/ 2/44	100
		5/ 2/44	100
5/ 3/44	90	5/ 3/44	90
		5/ 9/44	100
5/17/44	95	5/17/44	110

COMPARISON OF PHENOL COEFFICIENTS OBTAINED WITH CULTURES GROWN IN  
F. D. A. MEDIUM AND MEDIUM CONTAINING THE CASEIN HYDROLYZATE

Table 7 illustrates the results obtained in phenol coefficient tests obtained with *S. aureus* grown in F. D. A. medium and the experimental medium. Only those tests in which standard resistance (Ruehle and Brewer, 1931) was obtained are included in this table.

Two compounds, orthophenylphenol and chlorinated orthophenylphenol, were used to represent phenols of low and moderately high germicidal potency, respectively.

Variation arising from differences in pH of the phenol dilution under test was controlled by making up a large quantity of each test dilution of the sample. Aliquots of each dilution were then used for the phenol coefficient determination with each of the cultures grown in the two mediums. The sample dilutions were few enough so that both cultures were tested in the same experiment. These facts help to explain the close agreement between the results obtained with the two test mediums.

The variation obtained with organisms grown in the same medium but tested at different times is greater than the variation obtained in tests with organisms cultured in the two mediums.

RESISTANCE TO PHENOL OF *E. TYPHOSA* CULTURED IN THE MEDIUM CONTAINING  
A CASEIN HYDROLYZATE

Since *E. typhosa* is more widely used as a test organism than *S. aureus* in the evaluation of disinfectants, its resistance to phenol was studied after growth in the experimental medium. Table 8 illustrates the resistance of *E. typhosa* (A. T. C. C. 6539) to phenol obtained in three experiments.

Organisms grown in casein hydrolyzates, lots 46127 and 45076, had a resistance to phenol which was relatively consistent. After 10 to 15 transfers in these mediums, the resistance was less variable and the cultures retained the pearly sheen characteristic of cultures newly transferred to F. D. A. broth. The third lot, 44610, yielded organisms which were rather variable although this lot was prepared in a manner similar to the other two lots.

It is unfortunate that a study of the resistance of a culture grown in F. D. A. medium was not carried out parallel with the study of the experimental medium.

TABLE 8  
*Resistance to phenol of Eberthella typhosa cultured in experimental medium*

ORGANISM	LOT NO. OF CASEIN HYDROLYZATE	1:90 ++-	1:90 +--	1:95 ++-	1:95 +--	1:100 ++-	1:100 +--
A.T.C.C. no. 6539	0.76% 46127		6	9	25		
	0.76% 44610	3	17	1	4	10	1
	0.76% 45076		4	15	22		

However, it has been our experience that *E. typhosa* cultures grown in F. D. A. medium often become granular and resistant to phenol after continued transfer for two or more weeks. It is not uncommon to have cultures which survive in a 1:80 dilution of phenol for five or more minutes.

#### DISCUSSION

This work was initiated by the desire to eliminate some of the variation obtained in the testing of germicides by the F. D. A. test procedure. The official procedure requires that the test organism be transferred from an agar slant to F. D. A. broth at least once each month. Presumably this requirement is necessary in order to maintain standard resistance to phenol. However, the initiation of a broth culture every month from a new agar slant is a source of variation in itself as it is difficult to obtain exactly duplicate cultures of *S. aureus* from different slants. On the other hand, by using a reproducible liquid medium, cultures originating from the same source and of proper resistance can be maintained for relatively long periods of time. Indications obtained from work with the casein hydrolyzate are that continued transfer for six months would be entirely possible. One culture which has been transferred daily in the experi-

mental medium for about eight months has had a resistance reading of  $\pm$ — against phenol at dilutions of 1:60 or 1:65 in 140 of the 154 times in which resistances were determined.

The F. D. A. procedure also requires that test cultures of *S. aureus* must "survive a 1:60 dilution of phenol for 5 minutes and a 1:70 dilution for 15 minutes." The data already presented indicate that some variation in resistance is unavoidable. Furthermore, this variation occurs with cultures carried in either F. D. A. medium or in one of comparatively simple composition. However, the variation is not too great, and determinations made in this laboratory indicate that organisms giving a reading of  $\pm$ — against a 1:65 dilution of phenol give phenol coefficients within experimental range of those obtained with the organisms exhibiting the reading of  $\pm$ — in the presence of a 1:60 dilution of phenol. Those familiar with the testing of germicides will recall that it is not unusual to obtain readings of  $\pm$ — in the presence of phenol at 1:60 and  $+++$  or  $++$ — at a dilution of 1:70.

Therefore, it seems justifiable to consider whether or not the single resistance standard of the official test is too narrow. Certainly it has failed to accomplish the constancy of results desired. Greater latitude of the standard with insistence upon conformity of all broth cultures within its limits might well offer a more satisfactory criterion.

The variation in resistance of substrains of *S. aureus*, F. D. A. no. 209, now being used by different laboratories, introduces a variable quite as important as the medium in which the test organism is cultured. The differences in resistance to phenol shown by the nine substrains when checked in our laboratory under similar conditions undoubtedly reflects greater variations to be expected of tests carried out in different laboratories. These differences might be lessened if only one laboratory maintained the stock test culture. In this case those interested would turn directly to the central source for organisms as the need for renewed cultures arose. Since it has been shown that cultures of *E. typhosa* and *S. aureus* can be carried for extended periods of time with little variation, the inconvenience of obtaining cultures from a central laboratory would be outweighed by the assurance of working with an organism of reproducible resistance.

#### SUMMARY

A medium has been developed which contains acid-hydrolyzed casein and uracil as the main source of nitrogen. Cultures of both *Eberthella typhosa* and *Staphylococcus aureus* grown in this medium maintain a resistance to phenol equal to or greater than that obtained with currently available F. D. A. peptone medium.

Different lots of the casein hydrolyzate were found to serve equally well in supporting cultures with suitable and consistent resistance to phenol.

One substrain of *Staphylococcus aureus*, F. D. A. no. 209, has been transferred daily in the new medium for a period of eight months. Checks on the resistance of the organism to phenol have shown it to vary within relatively small limits.

Daughter cultures, grown in F. D. A. medium and the experimental medium and having the same resistance to phenol, gave the same results in phenol coefficient tests.

Nine substrains of *Staphylococcus aureus*, F. D. A. no. 209, were found to vary rather widely in their resistance to phenol when cultured in either F. D. A. medium or the medium containing the casein hydrolyzate.

Suggestions are offered to the effect that variations in germicidal testing other than those resulting from the immediate effect of differences in medium might be diminished by (a) the closer control and standardization of the test organism by a central laboratory, (b) the maintenance of test cultures in a reproducible liquid medium for periods extending into several months, and (c) the recognition of constancy of resistance as well as the attainment of a particular standard as a criterion in the evaluation of suitable test organisms.

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# A STUDY OF SOME ENVIRONMENTAL FACTORS WHICH CONTROL ENDOSPORE FORMATION BY A STRAIN OF *BACILLUS MYCOIDES*

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Much work has been done on the environmental factors which affect the formation of endospores by members of the genus *Bacillus*. The literature on this subject was adequately reviewed by Brunstetter and Magoon (1932). Other reports not mentioned by these authors, or which appeared since the publication of their paper, are those of Williams (1930-31), Cook (1931), Bayne-Jones and Petrilli (1933), Fabian and Bryan (1933), Roberts (1934), Greene (1938), Roberts and Baldwin (1942), and Hayward (1943). The environmental factors that have been investigated are the following: the temperature of incubation; the composition of the medium with respect to type and concentration of gross nutrients and salts (particularly cations, including the concentration of hydrogen ion), and the presence of nitrates; the accumulation of by-products of metabolism; the concentration of molecular oxygen; desiccation of the medium (Darányi, 1927); finally, the stage of development of the culture with reference to the "growth curve" (Henrici, 1928).

There seems to be agreement that the temperature interval for endospore formation is shorter than that for growth, that the minimum oxygen concentration for sporulation is higher than that for growth, and that optimum conditions for growth and sporulation are the same. There is little agreement on anything else. The old argument on whether the formation of endospores is due to exhaustion of the medium (Buchner, 1890) or whether it is caused by the accumulation of the by-products of metabolism (Lehmann, 1888; Migula, 1904) has not yet been settled. Having observed that the percentage of spores produced by *Bacillus mycoides* in aerated peptone solutions increased as the concentration of peptone decreased, and that the opposite was true for *Bacillus fusiformis*, Brunstetter and Magoon (1932) concluded that "both the amount of available food and the amount of metabolic products in the environment are of great importance in determining the extent of sporulation by *B. mycoides* . . .," whereas with *Bacillus fusiformis* "the accumulation of metabolic products seemed to be of much more importance than the food supply." The report of Cook (1931) that *Bacillus subtilis* forms spores only in the pH interval between 6 and 7 was contested by Fabian and Bryan (1933), who reported spore formation between pH 5.0 and 7.5. Williams (1929) was unable to get satisfactory spore yields with *Bacillus subtilis* in several synthetic media, whereas Roberts (1934) reported 60 to 70 per cent endospores of the same species, within 5 days, in a relatively simple synthetic medium, compared to less than 30 per cent in 1 per cent peptone solution. Hayward (1943) found no beneficial effect on the



sporulation of *Bacillus subtilis* from the addition of thiamine, nicotinic acid, pantothenic acid, riboflavin, pyridoxine, or biotin to nitrogen-free casein hydrolyzate; a slight stimulation was attributed to inositol.

The present investigation shows that most, if not all, of the environmental factors mentioned above are so interdependent that no true picture of the circumstances that control sporulation can be obtained without knowledge of this relationship.

#### ORGANISM AND TECHNIQUE

The organism used in this work is strain C<sub>2</sub> of *Bacillus mycoides* previously classified in this laboratory by Lamanna (1940).

In the early stages of this investigation, we used still and aerated liquid cultures, and the numbers of vegetative cells and endospores in a ml of culture were counted, after the proper dilution, by a modification of the method described by Knaysi and Ford (1938). Cultures intended for aeration had a volume of 40 ml and were grown in test tubes 260 mm long and 22 mm in internal diameter. Air was drawn by suction through glass tubes 4 mm in internal diameter. Cultures grown in the same medium were usually connected in series by means of rubber tubes. Sterility of the air was insured with cotton plugs in the glass and rubber tubes. In view of the fact that some of these experiments had to be run for a relatively long time, the air was passed through two bottles of distilled water, connected in series, before admission to the cultures, thus protecting the medium from evaporation. We also used slant cultures and estimated the relative numbers of vegetative cells and endospores in wet smears taken from the central area of the surface of the slant.

Although we gained considerable information from this early work, we soon realized that its results were both rough and limited in value. It is true that in aerated, liquid cultures environmental conditions are fairly uniform, but the great sensitivity of the process of sporulation to molecular oxygen requires a constant rate of aeration, which is very difficult to accomplish even in tubes connected in series. We have reasons to believe that wide variation in the rate of aeration may even be injurious. The inaccuracies of our method of estimating the proportions of vegetative cells and endospores in slant cultures are too obvious to merit discussion.

Our early experience indicated that there is no hope of getting reliable quantitative information on the relation of various factors, other than oxygen supply, to the process of sporulation except by eliminating the possibility of oxygen shortage. This meant the use of agar slant cultures under a relatively large air atmosphere. We also realized that uniform results can be obtained only when the medium is relatively dilute, not because a more concentrated medium is per se detrimental to the formation of endospores, as is stated by all students of the problem (cf. Henrici, 1928; Williams, 1930-31; Brunstetter and Magoon, 1932), but because greater concentration of nutrients means greater density of population and, consequently, less oxygen per cell; furthermore, complex culture media, particularly those containing sugars, compete with the cells for oxygen,

and this competition increases with the concentration of the medium. In the case of slant cultures, greater concentration of nutrients means a thicker layer of growth, and cells below the surface must either live anaerobically or enter a resting stage, or perish. In most of this work, therefore, we used the following medium: 100 ml of meat infusion of  $\frac{1}{4}$  strength + 0.25 g of tryptone + 1.5 g of agar; pH 7.0-7.2. When glucose was needed, 0.25 g of that sugar was added. The medium was slanted in 5 ml portions in test tubes 150 mm long and 22 mm in internal diameter. After hardening of the agar, the tubes were allowed to stay in the laboratory for a couple of days, and were then inverted and left 2 more days to remove any free liquid. They were then inoculated very lightly with a pure spore suspension of the organism; we were careful to spread the inoculum over the entire surface of the slant.

In studying the effect of moisture, the inoculated slant was inverted, after removal of the cotton plug, in a sterile, large test tube (205 mm long and 36 mm in internal diameter) over 5 to 8 ml of the moisture-controlling agent or solution, and was supported with a glass plate of the proper dimensions, so that the atmosphere of the culture tube and that of the outside tube were continuous. The outside tube was closed with a sterile rubber stopper.

Unless otherwise stated, all cultures were incubated at 33 C.

The numbers of vegetative cells and endospores in slant cultures were counted by a modification of the method described by Knaysi and Ford (1938). After receiving a definite volume of water, the slant was melted in flowing steam. In almost all instances, this heating followed by moderate shaking dispersed the vegetative cells and spores singly or in very small groups. A portion of the suspension was then used for colorimetric determination of pH, and another portion was used for counting, or for further diluting before counting; 0.01 ml of the proper dilution was placed on a glass slide and immediately covered with a square cover glass, 12 x 12 mm. The preparation was then very carefully sealed with "vaspar" (50 parts of vaseline + 50 parts of paraffin) to prevent currents. Diluting with the agar gelatin described by Knaysi and Ford would have made the use of vaspar unnecessary, but we found the present technique sufficiently accurate and more convenient. Similar preparations were also used for counting liquid cultures. In the latter case, we resorted to heating only when the culture was actively motile, or when the pellicle was not easily dispersed. In rare cases we had to use acid or alkali in order to obtain complete dispersion.

It is needless to emphasize that the preparations we used for counting are much superior to those involving drying, staining, differentiating, and restaining. Dried smears are never of uniform thickness, and repeated washing undoubtedly removes many vegetative cells and spores. Furthermore, spores in incipient germination, incompleated spores, and shells often indicate a definite condition in the culture; in stained smears, these would stain as vegetative cells or not at all. In our own preparations, these forms are recognizable, and all elements of the suspension are remarkably well distributed. Indeed, the greatest error of our method is that of sampling because of the minute volume measured and

the small bore of the pipette. Greater accuracy can probably be attained by the use of a 0.05 ml pipette of a relatively large bore, and of a larger cover glass. Table 1 shows the usual degree of agreement between duplicate preparations and recounts of the same preparation.

In view of the uniform distribution of cells and spores (table 2), the counting of only 10 fields was found adequate.

TABLE 1  
*Accuracy of the method of counting*

NO. OF EXPERIMENT	COUNTS OF THE SAME PREPARATION $\times 10^4$		COUNTS OF A DUPLICATE PREPARATION $\times 10^4$	
	Vegetative cells per ml	Endospores per ml	Vegetative cells per ml	Endospores per ml
I	333.6	9.6		
	352.8	12.0		
II	67.7	0.7		
	95.8	0.0		
III	49.0	0.0	39.0	0.0
IV	127.4	5.2	157.7	5.2
V	43.2	140.4	64.8	270.0
	36.0	136.8		
VI	26.4	75.6	14.4	79.2
VII	58.3	784.1		
	64.8	641.5		
VIII	1195.2	309.6	1022.4	180.0
			1209.6	151.2
IX	0.0	374.4		
	0.0	475.2		
X	229.0	7.2		
	219.0	8.6		

We are aware of the usual practice of reporting "percentage of spores." This practice may have some value but is misleading because it assumes that the vegetative cells present in the culture at a given moment represent all the cells that did not sporulate, just as the spores represent approximately all the cells that sporulated. The truth is that many vegetative cells disintegrate and disappear; in certain media and under certain conditions, the number of such cells is enormous. The products of this disintegration do not seem to be readily or completely utilizable by the other cells, and the number of vegetative cells at any given moment may be only a small fraction of what it would have been without disintegration. Consequently, we find that the absolute number per ml

of spores and vegetative cells at any given moment is of much greater significance. Those who are interested in the "percentage of spores" can compute it from the recorded numbers.

## EXPERIMENTAL

### 1. Evolution of Cultures

*a. Aerobic cultures.* The evolution of the cultures of strain C<sub>2</sub> of *Bacillus mycoides* used in the present investigation is similar to that recently described for *Bacillus subtilis* (Knaysi and Gunsalus, 1944). Ordinary, still aerobic cultures in broth, not especially buffered and not containing a fermentable sugar

TABLE 2

*Examples of the distribution of cells and spores in the microscopic preparations used for counting*

FIELD NO	PREPARATION 1			PREPARATION 2		
	Vegetative cells	Endospores		Vegetative cells	Endospores	
		Normal	Nonrefracting		Normal	Nonrefracting
1	12	3		12	10	
2	7	4		12	9	2
3	4	5		13	4	
4	15	3	1	10	6	
5	13	2		8	7	1
6	9	5	4	7	5	
7	5	6	1	12	5	
8	8	4		18	7	2
9	13	2		12	6	1
10	13	7		9	3	1
Average per field.....	9.9	4.1	0.6	11.3	6.2	0.7

(initial pH=7), become more and more alkaline until they reach a pH of about 9. In the presence of a fermentable sugar, aerobic broth cultures at first become more acid, reach a minimum pH the value of which depends on the medium, and then become more and more alkaline until they reach a maximum value of about pH 9. This shift to the alkaline side takes place probably as the sugar is used up, and as the acid by-products which consist mostly of lactic acid are further oxidized. In both cases, whether the culture initially did or did not contain a fermentable sugar, endospores are not formed appreciably until the culture has reached or, usually, exceeded pH 7.5 to 8.0 (table 3). In still, liquid cultures, the formation of endospores is related to that of a pellicle (table 11). In the absence of a pellicle, sporulation is considerably delayed and the number of endospores remains small. Broth cultures *aerated* by bubbling air undergo, qualitatively, a similar evolution; the early drop of pH in glucose-containing media may be slight and evanescent, but the subsequent rise of alkalinity is much slower than in cultures initially containing no fermentable

sugar. When a culture has reached its maximum alkalinity, further bubbling of air often results in a slow but consistent drop in the pH, more noticeable under conditions of vigorous aeration; this is probably due to further oxidation of basic by-products of metabolism, and causes a tendency to germination indicated by an increase in the number of endospores of low refractive index and, occasionally, by a reduction in the maximum number of normal endospores (table 4).

TABLE 3

*Evolution of still, liquid cultures of Bacillus mycoides, strain C<sub>2</sub>, at 33 C, in the indicated media and under the indicated conditions*

MEDIUM	CONDITION OF INCUBATION	AGE OF CULTURE <i>in days</i>	VEGETATIVE CELLS PER ML $\times 10^6$	ENDOSPORES PER ML $\times 10^6$		pH
				Normal	Non-refractive	
$\frac{\text{MITG}^*}{2}$ ; pH 7.0-7.2	Aerobic	1	124.6	0.0	0.0	5.4
		2	63.4	0.0	0.0	5.6
		3	81.7	0.0	0.0	6.4
		4	108.7	0.0	0.0	6.3
		5	166.3	0.7	0.0	6.8
		10	142.6	5.2	0.0	8.6
$\frac{\text{MITG}^*}{2} + 25 \text{ g MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; pH 7.0-7.2	Aerobic	1	287.3	0.0	0.0	5.6
		2	303.9	0.0	0.0	6.8
		4	596.2	19.4	0.0	7.6
		5	613.4	63.4	2.9	7.7
		7	201.6	178.6	54.7	8.0
		10	64.8	270.0	79.2	8.8
MITG*; pH 7.0-7.2	Anaerobic (vaspar seal)	1	44.8			6.4
		2	13.4			6.4
		3	0.5			6.2
		5	1.9			6.0
		8	0.7			5.8

\* MI = 100 ml of meat infusion; T = 1 g of tryptone; G = 1 g of glucose. The denominator indicates the dilution.

In *slant cultures* of infusion agar with or without a fermentable sugar, the evolution is fundamentally similar to that which takes place in liquid media, but may be obscured by the spacial relation between the cells and their food supply. On the surface of a slant the growth rate is so rapid and the population so dense that the melted slant gives almost maximum alkalinity within one day under the conditions of the present experiments (tables 6 and 8). However, it is possible to see in young cultures that the layer next to the cells gets alkaline long before the rest of the slant.

*b. Anaerobic cultures.* Strain C<sub>2</sub> of *Bacillus mycoides* is unable to grow anaerobically to a noticeable extent in tryptone solutions, with or without meat

infusion, unless a fermentable sugar is added. This is strikingly shown in the following experiment: A suspension of washed endospores was prepared in a 0.5 per cent tryptone solution; it contained  $7.2 \times 10^6$  normal endospores and

TABLE 4

*Evolution of aerated cultures of Bacillus mycoides, strain C<sub>2</sub>, at 33 C in the indicated media and under the indicated conditions*

MEDIUM	RATE OF AERATION; BUBBLES PER SECOND	AGE OF CULTURE	VEGETATIVE CELLS PER ML $\times 10^6$	ENDOSPORES PER ML; $\times 10^6$		pH
				Normal	Non- refractive	
$\frac{\text{MITG}^*}{2}$ ; pH 7.0-7.2	1-2	<i>in days</i>				
		6	296.2	0.0		7.6
		10	168.0	9.6		
		15	80.0	12.0		8.5
$\frac{\text{MITG}^*}{2}$ + 25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; pH 7.0-7.2	1-2	21	9.9	40.3		8.7
		6	132.7	0.0		6.4
		10	215.0	3.2		
		15	346.0	12.0		7.3
$\frac{\text{MIT}^*}{2}$ ; pH 7.0-7.2	2-4	21	23.8	54.4		9.0
		1	492.0	4.8	0.0	7.9
		2	249.6	21.6	0.0	8.7
		4	288.0	21.6	0.0	9.2
		6	37.9	26.4	0.0	9.1
		11	20.6	54.2	10.6	8.5
$\frac{\text{MITG}^*}{2}$ ; pH 7.0-7.2	2-4	15	0.9	36.0	16.8	8.1
		1	381.6	0.0	0.0	7.2
		2	204.0	0.0	0.0	7.8
		4		2.4	0.0	8.7
		6	67.8	79.2	0.0	
		11		72.0	14.4	9.2
$\frac{\text{MIT}^*}{4}$ ; pH 7.0-7.2	3-10	15		115.2	31.2	8.8
		1	54.0	0.0	0.0	7.8
		3	32.9	1.4	0.0	8.6
$\frac{\text{MITG}^*}{4}$ ; pH 7.0-7.2	3-10	6	23.2	29.6	2.8	8.5
		1	364.8	0.0	0.0	6.7
		3	162.0	31.2	3.6	8.6
$\frac{\text{MITG}^*}{4}$ ; pH 7.0-7.2	3-10	6	87.1	23.3	2.4	8.2

\* MI = 100 ml of meat infusion; T = 1 g of tryptone; G = 1 g of glucose. The denominator indicates the dilution.

$3.8 \times 10^6$  nonrefractive ones per ml. At the end of 4 hours at about 25 C, the suspension was examined microscopically in a sealed preparation; it showed considerable typical germination (strictly equatorial with splitting of the exine). At the end of 22 hours the same preparation was reexamined and appeared very

much as it did at the end of 4 hours, i.e., it consisted mostly of germinating spores and germ cells still attached to the spore coat. The suspension from which the preparation was made had in the meantime become turbid and formed a pellicle.

In the presence of glucose, the organism grows anaerobically (although the population remains relatively low) and the pH of the culture gradually drops to about 5.8 (table 3). In meat-infusion glucose broth the organism is unable to oxidize the acids formed, and the pH remains at the minimum reached. No endospores are formed in *strictly anaerobic* cultures even when they are several months old (vaspar seals are not effective when the period of incubation is thus extended; occasional spores have also been observed by the author in such cultures of *Bacillus subtilis* over 3 months old, although the pH was still at its minimum level).

## 2. The Effect of Oxygen

It has been pointed out in the previous section that endospores are not formed under *strictly anaerobic* conditions. On the other hand, we found endospores in very old glucose infusion cultures of *Bacillus alerimus* (and several other members of the genus *Bacillus*) incubated under vaspar seals. Under vaspar and oil seals, the conditions remain anaerobic as long as the supply of reducing material in the medium is not exhausted. This observation indicates, but does not prove, the necessity of molecular oxygen for the formation of endospores. On the other hand, the necessity of oxygen for the quick and efficient formation of large numbers of endospores is one of the incontrovertible facts of bacteriology.

It was not the purpose of this work to make a complete study of the relationship between oxygen and sporulation. Consequently this phase of the work was developed only to the extent it contributed to an understanding of the general problem.

Our preliminary work with *aerated cultures* (table 4) shows that when the rate of aeration is slow, the formation of endospores is slow and their numbers remain relatively small. As the rate of aeration increases, endospores are formed sooner and in greater numbers. The same is true of the vegetative population. This effect of oxygen on growth is of course due to a considerable increase in the total available energy of the medium and in the rate at which it can be utilized. If the pH of the culture be taken as an index of the extent of utilization of food material, a glance at table 4 shows that doubling the rate of aeration results in more than doubling the rate of food utilization.

Table 4 also shows that when glucose (0.5 per cent) is present in the medium, a slow rate of aeration results in an extension of the period of purely vegetative growth. Sporulation may be hastened either by a reduction in the concentration of glucose or by an increase in the rate of aeration, and, unless the concentration of sugar is sufficiently low, it may be difficult to aerate with sufficient vigor to promote sporulation within a reasonable time. We have evidence that wide fluctuations in the rate of aeration are detrimental both to the formation of the endospores and to their preservation. Increasing the rate of aeration

may make possible more complete oxidation, which is accompanied by a tendency to germinate.

The two outstanding lessons learned from this early work are (1) that sporulation is extremely sensitive to oxygen and (2) that even vigorous aeration does not usually supply the culture with all the oxygen it can utilize. The latter fact is particularly true for glucose media and is strikingly illustrated in table 5. In this experiment, the two tubes were connected in series; the first contained the usual 40 ml of inoculated medium, and the second 3 ml of the same medium; the concentration of the inoculum per ml was the same in both tubes. Our notes indicate an aeration sufficiently vigorous (about 10 bubbles per second) to transform the entire 3 ml into foam. The tremendous difference in the number of spores here is also correlated with the degree of depletion of the medium as indicated by the pH.

In the light of this experience, and in view of the practical difficulty of realizing a constant aeration, we concluded that it is hopeless to investigate the effect of

TABLE 5

*Experiment with an aerated culture showing the effect of foaming on the formation of endospores by strain C<sub>2</sub> of Bacillus mycoides at 33 C and the conditions indicated*

MEDIUM	AGE OF CULTURE	PHYSICAL STATE OF CULTURE	VEGETATIVE CELLS PER ML $\times 10^6$	ENDOSPORES PER ML $\times 10^6$		pH
				Normal	Nonrefractive	
	<i>in days</i>					
MITG*	1	Liquid	326.4	0.0	0.0	7.4
4; pH	1	Foam	290.4	172.8	0.0	8.3
7.0-7.2						

\* MI = 100 ml of meat infusion; T = 1 g of tryptone; G = 1 g of glucose. The denominator indicates the dilution.

any factor on sporulation unless the oxygen supply is kept constant or practically so, and we decided to resort to the use of *slant cultures*. By keeping the volume of the medium, the free surface of the slant, and the inoculum approximately constant, results can be duplicated with reasonable accuracy provided the air atmosphere is sufficiently large. The required volume of that atmosphere obviously depends on the volume and composition of the medium. In test tubes 150 mm long and 15 mm in internal diameter closed with a rubber stopper, a 5-ml slant culture of a medium containing 100 ml of meat infusion ( $\frac{1}{2}$  strength) + 0.5 g of tryptone + 0.5 g of glucose + 1.5 g of agar practically contains no endospores; the relatively few spores formed owing to temporary shortage of food, before the available oxygen is consumed, germinate as more nutrients diffuse to the surface, and only a few shells can be observed in such cultures. In view of these results, the minimum oxygen pressures reported for endospore formation must be taken with caution, unless it is shown that the recorded initial pressure remained constant at the time of sporulation; otherwise, the usual statement that the minimum oxygen pressure for endospore formation



(in media which do not allow anaerobic growth) is higher than that for growth would have no meaning.

### 3. The Effect of $MgSO_4$

Magnesium sulfate is known to cause morphological changes in *Bacillus mycoides* (Stapp and Zycha, 1931). As far as we know, however, no one has reported an effect of this salt on the formation of endospores. Fabian and Bryan (1933), working with *Bacillus subtilis*, *Bacillus mesentericus*, *Bacillus cereus*, and *Bacillus megatherium*, did not observe any stimulation of reproduction or of spore formation in a liquid medium due to  $MgCl_2$ .

In the course of a different investigation, we made the following observation: When *Bacillus mycoides* (strain C<sub>2</sub>) is inoculated into glucose broth containing  $MgSO_4$  in a relatively high concentration (100 ml of meat infusion + 1 g of tryptone + 1 g of glucose, diluted with an equal volume of  $MgSO_4$  solution prepared by dissolving 50 g of  $MgSO_4 \cdot 7H_2O$  in 100 ml of water; pH 7.0-7.2), the organism grows at first throughout the medium, then a pellicle begins to form as the turbidity of the culture tends to disappear. Further incubation results in an increase in the thickness and toughness of the pellicle and in the almost complete disappearance of turbidity from the body of the medium. At about the fifth day (33 C; the time varies somewhat with amount of inoculum and temperature), turbidity reappears in the medium and may reach a considerable intensity, and the increase in turbidity is accompanied by disintegration of the pellicle. Microscopic examination reveals that the turbid liquid is a heavy suspension of almost pure endospores. In the absence of  $MgSO_4$ , the early sequence of events is similar, but there is no reappearance of turbidity; sporulation in the pellicle is slow, and the endospores never reach a high number; the pellicle is more perishable and does not have sufficient adhesion power to the walls of the tube. Careful counts revealed that  $MgSO_4$  results not only in a tremendous increase in the number of spores (which is comparable to what one gets on slants) but also a severalfold increase in the number of vegetative cells (table 3).

In following up this apparently important observation, we found that the effect of  $MgSO_4$  on the total population is also observed, although to a lesser degree, both in aerated liquid cultures and in slant cultures. The same was true for the effect of this salt on the number of endospores in aerated cultures (table 4), but no increase in the number of endospores was noticeable in slant cultures; on the contrary, a definite decrease was usually observed (table 6). We have not yet sufficiently studied the mechanism of action of this salt; however, there seems little doubt that it is partly physiological. The effect on sporulation seems to be correlated with the availability of molecular oxygen, the stimulation becoming less as molecular oxygen is more plentiful. It can also be noticed that, in the presence of  $MgSO_4$ , sporulation seems to begin at a slightly lower pH. In the case of liquid, still cultures, we believe that the strong stimulation is due, not only to a physiological action of the salt, but also to the luxurious surface growth where molecular oxygen is plentiful.

4. *The Effect of Starvation*

In order to test whether endospores are formed by normal cells faced with starvation, experiments similar to the following were performed:

Cells were centrifuged out from aerated cultures at various ages of those cultures; the cellular sediments were resuspended, without washing,

TABLE 6

*Effect of  $MgSO_4$  on the formation of endospores by strain C<sub>2</sub> of *Bacillus mycoides* at 33 C and the conditions indicated*

MEDIUM	AGE OF CULTURE  in days	VEGETATIVE CELLS PER ML; $\times 10^6$	ENDOSPORES PER ML; $\times 10^6$		pH
			Normal	Non-refractive	
$\frac{MITG^*}{2}$ + 1.5 g agar	1	418.3	504.0	15.1	9
	2	61.6	712.8	29.2	8.8
$\frac{MITG^*}{2}$ + 12.5 g $MgSO_4 \cdot 7H_2O$ + 1.5 g agar	1	1028.2	247.0	10.1	8.4
	2	359.0	486.0	21.6	8.7
$\frac{MITG^*}{2}$ + 1.5 g agar	1	377.8	284.4	21.6	8.8
	2	93.6	928.8	14.4	8.8
	3	28.8	871.2	43.2	9.0
	5	64.8	1317.6	79.2	9.0
$\frac{MITG^*}{2}$ + 12.5 g $MgSO_4 \cdot 7H_2O$ + 1.5 g agar	1	1142.4	213.6	24.0	8.4
	2	540.0	1022.4	21.6	8.8
	3	208.8	720.0	50.4	8.9
	5	136.8	727.2	295.2	9.2
$\frac{MITG^*}{4}$ + 1.5 g agar	1	90.0	529.2	28.8	8.7
	2	32.4	730.8	43.2	8.8
	3	12.6	500.4	27.0	8.8
	4	21.6	633.6	43.2	8.8
	8	0.0	424.8	19.8	8.6
$\frac{MITG^*}{4}$ + 12.5 g $MgSO_4 \cdot 7H_2O$ + 1.5 g agar	1	295.2	504.0	3.6	8.6
	2	129.6	439.2	18.0	8.8
	3	27.0	329.7	39.6	9.0
	4	39.6	396.0	32.4	9.0
	8	1.8	244.5	59.8	8.8

\* MI = 100 ml of meat infusion; T = 1 g of tryptone; G = 1 g of glucose. The denominator indicates the dilution. pH = 7.0-7.2.

in sterile, distilled water, and the suspensions were aerated by bubbling air at approximately the same rates as the mother cultures. The results are illustrated in table 7. These results show that exposure of the cells to starvation hastens the formation of endospores, and more quickly when the mother culture is mature

than when it is young. Formation of endospores in distilled water has also been previously reported by Buchner (1890) and by Schreiber (1896).

### 5. Effect of the Concentration of the Medium

Williams (1930-31) reported that when *Bacillus subtilis* is grown in shallow layers of peptone solutions (1 to 5 per cent), the percentage of spores varied inversely with the concentration of peptone, although the total number of spores was higher in the more concentrated solutions. Brunstetter and Magoon (1932) also reported that when *Bacillus mycoides* is grown in aerated peptone solutions, the percentage of spores at the end of one day decreased as the concentration of peptone increased. These observations can find confirmation

TABLE 7

*Experiments with aerated cultures showing the effect of starvation on the formation of endospores by Bacillus mycoides, strain C<sub>2</sub>, at 33 C and the conditions indicated*

INITIAL MEDIUM OF THE MOTHER CULTURE	AGE OF MOTHER CULTURE AT TIME OF CENTRIFUGING	CENTRIFUGED, UNWASHED CELLS SUSPENDED IN	AGE OF AERATED SUSPEN- SION	VEGETA- TIVE CELLS PER ML $\times 10^6$	ENDOSPORES PER ML $\times 10^6$		pH
					Normal	Nonrefr.	
	<i>in days</i>		<i>in hours</i>				
MITG* 4 7.0-7.2	1			326.4	0.0	0.0	7.4
		Dist. water	4.5	139.2	0.0	0.0	7.7
		Dist. water	22.0	36.0	19.2	0.0	8.6
	2			235.0	0.0	0.0	6.5
	3			295.2	2.4	0.0	7.0
MITG* 4 7.0-7.2		Dist. water	4.5	131.4	6.6	0.0	8.1
	3			75.6	12.0	0.0	8.2
		Dist. water	0.0	49.7	8.6	0.0	
		Dist. water	6.0	40.3	23.8	0.0	
	5			42.0	22.8	0.0	8.3

\* MI = 100 ml of meat infusion; T = 1 g of tryptone; G = 1 g of glucose. The denominator indicates the dilution.

in table 8. We believe, however, that the conclusions of those authors were stated in a way which tends to obscure the relationship between sporulation and concentration of the nutrients.

It has been pointed out in the section on technique that the percentage of spores is not necessarily a measure of the tendency to sporulate. When two slant cultures initially differ only in the concentration of nutrients, the one with the dilute medium will sporulate sooner than the one with the more concentrated medium and, if the time interval at the end of which the cultures are examined is sufficiently short, the culture with the dilute medium will contain a higher number of spores; at the end of a longer interval, it may have a smaller number, but a higher percentage, of spores. As the time interval is increased, the percentage of spores in both cultures tends to become equal. This statement is limited to concentrations in which growth is not slowed down either by deficiency

or by toxicity. Assuming that the oxygen supply is not a factor, the percentage of spores is a measure of the extent to which nutrients have been used up.

Two questions may be asked in connection with the concentration of nutrients: the first is whether there is a minimum concentration below which sporulation is not possible; the second is whether there is a quantitative relation between concentration of nutrients and number of spores. A glance at table 8 shows that endospores can be formed on 1.5 per cent bacto agar, prepared with distilled water, to which no nutrients have been added. Table 8 also shows that in dilute media the number of endospores is proportional to the concentration of nutrients.

TABLE 8

*Effect of the concentration of nutrients on the formation of endospores by strain C<sub>2</sub> of Bacillus mycoides at 33 C and the conditions indicated*

MEDIUM	AGE OF CULTURES  <i>in days</i>	VEGETATIVE CELLS PER ML; × 10 <sup>8</sup>	ENDOSPORES PER ML; × 10 <sup>6</sup>		pH	REMARKS
			Normal	Non- refractive		
MIT* 8 + 1.5 g agar pH 7.0- 7.2	2	23.8	203.0	4.3	8.8	
	4	19.4	207.4	2.2	9.0	
MIT* 4 + 1.5 g agar pH 7.0- 7.2	1	42.6	181.0	3.0		Average of 4 experiments
	2	45.9	404.0	6.2		
MITG* 4 + 1.5 g agar; pH 7.0-7.2	1	263.1	615.6	18.0	8.6	Average of 5 experiments
	2	80.8	1300.0	42.4	8.8	
MITG* 2 + 1.5 g agar; pH 7.0-7.2	1	398.1	393.8	18.4	8.9	Average of 2 experiments
	2	77.6	820.8	21.8	8.8	
	5	64.8	1317.6	79.2	9.0	
1.5 g of bacto agar + 100 ml of dist. water	4	1.44	5.28	0.7	7.6	In sporangia

\* MI = 100 ml of meat infusion; T = 1 g of tryptone; G = 1 g of glucose. The denominator indicates the dilution.

Beyond a certain concentration, the number of endospores increases with the concentration but is not proportional to it. Barring toxicity, which has been often reported for concentrated peptone solutions, the reason for this apparently harmful effect of concentration must be sought in the fact that, for a given slant surface, the more concentrated slant gives a thicker layer of cells; below the surface of this layer, the environment varies from semiaerobic to anaerobic and, therefore, is unsuitable for sporulation. It can be easily shown that the numbers of endospores and of vegetative cells in slant cultures of the same initial composi-

tion are, at least within a certain period, functions of the ratio between the free surface area and the volume of a slant; this point should be kept in mind when slants are used in sporulation studies.

### 6. The Effect of Drying

It is natural to think of drying as a factor favorable for the formation of endospores. It is known, for instance, that maturation of the endospore is accompanied by shrinkage, and the favorable environment of slant surfaces, foam, and of unsubmerged pellicles is suggestive of a favorable effect of drying. The difficulty of obtaining an early and good spore yield in aerated, liquid cultures

TABLE 9

*Effect of drying on the formation of endospores by strain C<sub>2</sub> of Bacillus mycoides at 33 C and the conditions indicated*

MEDIUM	CULTURE INCUBATED OVER	AGE OF SLANT CULTURE	VEGETATIVE CELLS PER ML; $\times 10^6$	ENDOSPORES PER ML $\times 10^6$	
				Normal	Nonrefractive
MITG* 4 + 1.5 g of agar; pH 7.0-7.2	0.1 NNaOH	<i>in days</i> 1	34.8	112.8	1.2
		2	26.4	375.2	8.0
	70% NaOH	1	15.6	123.6	3.6
		2	34.8	319.9	2.4
	0.1 NNaOH	1	48.6	257.0	3.6
		2	79.2	486.0	3.6
	70% NaOH	1	70.6	230.4	3.6
		2	43.2	435.6	0.0
	0.1 NH <sub>2</sub> SO <sub>4</sub>	1	55.8	136.8	0.0
		2	50.4	482.4	21.6
	Conc. H <sub>2</sub> SO <sub>4</sub>	1	86.4	154.8	1.8
		2	79.2	324.0	10.8

\* MI = 100 ml of meat infusion; T = 1 g of tryptone; G = 1 g of glucose; pH 7.0-7.2.

also suggests the lack of opportunity for drying as a cause. Consequently, it is not surprising that Darányi (1927) attributed to drying an important role in the formation of endospores.

In the present work, one can find proof that drying is not necessary for the formation of endospores; for instance, sporulation under a vaspar seal and in cultures aerated with moist air. We also ran numerous experiments to find out whether drying, although not necessary, may be helpful. We used agar slant cultures incubated in an atmosphere where the moisture content was regulated with solutions of NaOH and H<sub>2</sub>SO<sub>4</sub> (table 9). The atmosphere was sufficiently large to eliminate oxygen as a factor (see the section on technique). Comparison

with similar slant cultures plugged with cotton convinced us that the latter precaution was effective.

The data of table 9 show that, under the conditions of our experiments, drying exerts a harmful effect. Only once did we find an increase, due to drying, in the number of endospores in a slant culture of glucose agar, and this effect may have been indirect. If the surface of a glucose agar slant was initially covered with a film of free water, drying may be beneficial in reducing the opportunity for anaerobic growth. It is also possible that the harmful effect of drying is also indirect and due to a depressing influence on the diffusion of nutrients and of by-products of metabolism. Our data show, however, that drying is not usually of much importance as a factor in the formation of endospores by strain C<sub>2</sub> of *Bacillus mycoides*.

The important role attributed to drying by Darányi (1927) in the sporulation of *Bacillus subtilis* and *Bacillus anthracoides* is obviously due to a greater availability of oxygen. This can be readily deduced from his statement that, of the various methods he used (alcohol vapor, CaCl<sub>2</sub>, etc.), the best results were obtained by drying on glass surfaces for two days.

### 7. The Effect of pH

It has been pointed out in a previous section that there is disagreement among investigators regarding the cardinal pH points for endospore formation by members of the genus *Bacillus*. We have repeatedly observed spores in old liquid cultures of various species at their respective minimum pH levels under vaspar seals; in the case of *Bacillus atterimus*, at pH 5.4. We have also seen that *Bacillus mycoides* forms spores at pH levels > 8. Those values are for the environment and not for the inside of the cell. Indeed, the intracellular pH depends not only on the pH of the medium but also on the material responsible for the pH; the equilibrium is determined by the permeability of the cell membranes.

In determining the optimum pH for sporulation, we used mixtures of equimolar KH<sub>2</sub>PO<sub>4</sub> and K<sub>2</sub>HPO<sub>4</sub> solutions in varying proportions. In order to keep the pH nearly constant in spite of growth, we had to use a very dilute medium. The composition of the medium and other data are given in table 10. The tubes containing the slant cultures were plugged with cotton.

The results indicate (table 10) that, under the conditions of the present experiments, there is a definite optimum pH for the sporulation of the investigated organism. This optimum lies in the interval 6.6 to 6.8.

As the pH increases, its unfavorable effect on sporulation quickly becomes noticeable. The number of endospores drops sharply as one passes the neutral point, although the total population may not be materially reduced. It is possible that at alkaline pH there is accumulation of some harmful by-product which may not stop vegetative growth but may render many cells too weak to form endospores.

Therefore, it may be concluded that endospores may be formed within a wide range of pH by the organism studied, with an optimum within the narrow interval of 6.6 to 6.8 under the conditions of the present experiments.

### 8. Effect of the Accumulation of By-products

Study of the effect of the by-products of metabolism on the formation of endospores is difficult, because it is difficult to separate by-products from the environment in which they are formed. In glucose broth cultures, hydrogen ions accumulate only when there is shortage of oxygen; the concentration of hydroxyl ions is proportional to the depletion of the medium.

In the course of other studies we observed that the organism investigated can grow in a solution of ammonium sulfate, glucose, and phosphate (table 11).

TABLE 10

*Effect of pH on the formation of endospores by strain C<sub>2</sub> of Bacillus mycoides at 33 C and the conditions indicated*

MEDIUM	pH		AGE OF CULTURE  <i>in days</i>	VEGETATIVE CELLS PER ML; $\times 10^6$	ENDOSPORES PER ML $\times 10^6$	
	Initial	Final			Normal	Nonrefractive
MIT* 8 + about 0.5 g phosphate mixture + 1.5 g of agar	5.6	6.0	1	15.2	45.4	18.7
		6.3	2	9.6	46.8	28.8
	6.4	6.4	1	20.2	86.4	9.4
		6.6	2	12.0	78.0	20.4
	6.6	6.8	1	14.4	109.4	7.2
		6.8	2	2.4	111.6	31.2
	7.0	7.0	1	49.7	56.9	5.0
		7.2	2	7.2	86.4	19.2
	7.4	7.5	1	81.4	34.6	5.0
		7.5	2	45.9	48.0	19.2
	7.8	8.0	1	71.3	29.5	4.3
		8.0	2	30.0	21.6	7.8

\* MI = 100 ml of meat infusion; T = 1 g of tryptone. The denominator indicates the dilution.

In such a solution, the pH gradually dropped so that in 3 to 4 days at 33 C it reached a minimum of pH 5.4; at about this time also, the maximum population was reached and a pellicle formed; at the end of 10 days, the pH was still 5.4, the population considerably reduced, but no endospores were observed either in the pellicle or elsewhere.

Working with agar slant cultures of the same medium, it was found that the pH of the culture dropped to 5.6 and was still 5.8 after almost two days when relatively few spores had been formed; the maximum number of spores was reached in 5 days. The low pH reached in slant cultures of this medium was surprising and suggested a possible shortage of vitamin B<sub>1</sub> for further oxidative decarboxylation of the organic acids formed from the sugar. Indeed, when the

TABLE 11

*Effect of acid by-products on the formation of endospores by strain C<sub>2</sub> of Bacillus mycoides at 33 C and the conditions indicated*

MEDIUM	AGE OF CULTURES		VEGETATIVE CELLS PER ML: $\times 10^6$	ENDOSPORES PER ML: $\times 10^6$		FINAL pH	REMARKS
	Days	Hours		Normal	Nonrefracting		
0.2 g (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> + 0.2 g glucose + 0.2 g phosphate mixture + 100 ml dist. water; pH 6.8*	0	0	0.0	6.2	0.1	6.8	
	1	0	5.2	3.4	0.5	6.8	
	3	0	26.2	4.1	1.4	6.0	Pellicle
	4	0	30.2	3.4	1.0	5.4	Pellicle + ring
	10	0	8.4	1.2	1.0	5.4	Pellicle + ring
0.2 g (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> + etc. as above + 1.5 g agar; pH 6.6	0	22	414.0	0.0	0.7	5.6	
	1	17	111.8	9.4	0.0	5.8	
	5	0	20.2	61.4	10.6	6.4	
	19	0	7.9	44.0	12.2	6.4	
0.2 g (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> + etc. as above + 1.5 g agar + 10 $\mu$ g vitamin B <sub>1</sub> ; pH 6.3	0	22	214.1	5.3	3.4	6.4	
	1	17	70.8	139.2	30.5	6.8	
	5	0	8.6	358.6	41.8	7.5	
	19	0	0.0	216.0	69.1	6.8	
0.1 g vitamin-free casein hydrolyzate + 0.2 g phosphate mixture + 100 ml dist. water; pH 6.9	0	0	0.0	2.6	0.0	6.9	
	1	0	2.2	0.5	0.5	6.8	
	2	0	15.6	0.0	1.9	6.8	
	3	0	26.4	0.5	2.4	6.8	In sporangia
	6	0	20.9	0.0	0.7	7.0	No pellicle
	10	0	1.0	38.4	3.6	8.4	Pellicle
0.1 g vitamin-free casein, etc. as above, + 0.2 g glucose; pH 6.8	0	0	0.0	2.6	0.0	6.8	
	1	0	25.0	0.0	1.0	6.4	
	2	0	21.0	0.0	0.0	5.4	Pellicle
	3	0	26.9	0.0	0.0	5.6	Pellicle
	6	0	57.1	0.2	0.7	6.4	Pellicle
	10	0	24.2	14.2	1.0	7.4	Pellicle
0.1 g vitamin-free casein, etc. as above, + 0.2 g glucose + 1.5 g agar; pH 6.8	2	0	79.9	226.8	36.7	7.0	
	4	0	15.6	210.8	53.6		
0.1 g vitamin-free casein, etc. as above, + 0.2 g glucose + 10 $\mu$ g vit. B <sub>1</sub> + 1.5 g agar; pH 6.8	2	0	54.0	246.2	0.0	7.4	
	4	0	64.8	200.9	17.3		

\* One-month-old Eldredge tube cultures in this medium, distributed in shallow layers, showed some sporulation.

vitamin was incorporated to the extent of 10 $\mu$ g per ml, no drop in the pH was observed at the end of the time intervals indicated in table 11. The vitamin seemed to have no beneficial effect on vegetative growth, but endospores were



formed earlier and their maximum number was about 6 times greater than in cultures to which the vitamin was not added.

It was now a question of whether the beneficial effect of vitamin B<sub>1</sub> on sporulation was due to the quick removal of the acid by-products or to a specific role in the process of sporulation.

Experiments with agar slant cultures in which the (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was replaced by vitamin-free casein hydrolyzate showed that the pH of those cultures does not drop even when vitamin B<sub>1</sub> is not added, and that the vitamin has no beneficial effect on sporulation.

In view of these results, the relatively small number of spores formed in the (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> medium to which no vitamin was added must be attributed to the persistence of a low pH. The addition of vitamin B<sub>1</sub> makes the environment more suitable for sporulation by removing the acid by-products of glycolysis. The excellent vegetative growth of the organism in this medium together with the slow rise in the pH of the culture leads to the conclusion that vitamin B<sub>1</sub> is synthesized in this medium, although not with sufficient speed. It is probable that in the casein hydrolyzate medium, the vitamin is readily synthesized so that the pH of the culture does not drop to a level which would injure the cells and hinder sporulation.

#### DISCUSSION AND CONCLUSIONS

In studying the environmental factors which affect the formation of endospores, the various investigators have dealt with each factor as an independent variable. In reality, those factors are so interrelated that the proper evaluation of any one factor is not possible unless its relation to other factors is elucidated. This requires familiarity with the physiological behavior of the organism investigated.

In the case of strain C<sub>2</sub> of *Bacillus mycoides*, and indeed of a number of other members of the genus *Bacillus* with which we are familiar, it is important to know that the temperature, the concentration of oxygen, the state and density of the population, the utilization of nutrients, the accumulation of the by-products of metabolism, the spontaneously developed pH, etc., are intimately interrelated. In the presence of a fermentable sugar, the organism can grow anaerobically, but the acid by-products of glycolysis, chiefly lactic acid, accumulate and the pH drops to a minimum which varies with the medium; under strictly anaerobic conditions, endospores are not formed. Now what is the limiting factor? Is it the absence of oxygen or is it the accumulation of the acid by-products? When oxygen is plentiful, oxidation is usually immediate and complete; the pH drops only slightly and then rises to a maximum. Under these conditions endospores are formed, but only as the alkalinity of the culture has considerably risen. Now what caused the formation of endospores? Is it the presence of oxygen, or the alkalinity, or the exhaustion of the nutrients? Spores are formed best on the surface of a solid medium. Is it because of greater availability of oxygen or is it because of gradual drying? All those questions must be answered before a clear picture of the conditions necessary, helpful, or detrimental for sporulation are clearly known.

In this paper it has been shown that the accumulation of the acid by-products of glycolysis is harmful for sporulation. It must be added that the accumulation of hydroxyl ions in sporulating cultures is not the cause of endospore formation: first, because an imposed alkaline reaction is detrimental; and, second, because when portions of old, aerated cultures which have reached their maximum alkalinity are neutralized, diluted up to 5 times, or both, the spores already formed do not seem to germinate. The concentration of hydroxyl ions merely indicates the extent to which the nutrients have been used up. On the other hand, although endospores are not formed under strictly anaerobic conditions, it has been shown that a certain number is formed in very old cultures (of *Bacillus aterraimus* which is physiologically similar to *Bacillus mycoides*) under a vaspar seal, even at the minimum pH of the culture, which means in the presence of the acid by-products of glycolysis at their maximum concentrations. This indicates, but does not prove, that in endospore formation there may be at least one process that involves reaction with molecular oxygen. Nevertheless, no matter how plentiful oxygen is, spores are not formed until the sugar and the products of glycolysis are completely oxidized and the nitrogenous components of the medium are largely used up, as indicated by the evolution of the pH. Aside from the possible necessity of oxygen per se, its chief role seems to be the promotion of a rapid and complete utilization of the nutrients and, as a corollary, the prevention of the accumulation of by-products which may injure the cells and tend to inhibit sporulation. It has been shown in a previous section that neither acid nor basic by-products can greatly accumulate when oxygen is plentiful.

In the present work we have not made any special study of the nutritive requirement of the strain investigated; however, we were able to grow the organism in an extremely simple synthetic medium and in vitamin-free casein hydrolyzate (table 11). In view of the fact that no special precautions were taken to insure the absence of nutritives from the distilled water or the chemicals used, it may only be concluded that the nutritive requirements of the organism, for growth as well as for sporulation, are either nil or very slight. On the other hand, we have shown that growth factors, as illustrated by vitamin B<sub>1</sub>, may exert an indirect effect under special conditions. We have shown that a greater number of spores may be obtained when vitamin B<sub>1</sub> is added to a medium in which the acid by-products of glycolysis tend to accumulate (the ammonium sulfate medium). By performing its function as a cocarboxylase, the vitamin tends to promote further oxidation of those by-products and to prevent a considerable drop in the pH. In a medium where the acid by-products of glycolysis do not accumulate (the casein-hydrolyzate agar medium), addition of the vitamin is of no value. It is possible that in the casein hydrolyzate medium the vitamin is synthesized much more readily than in the (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> medium. Reports by nutritionists of the stimulating but unnecessary presence of a certain nutritive may be due to such an indirect effect in an environment where the synthesis of that nutritive is slow or difficult.

The only possible conclusion to be drawn from this work with strain C<sub>2</sub> of *Bacillus mycoides* is that endospores are formed in an aerobic environment by healthy cells facing starvation. Besides all the evidence obtained from cultures,

the fact that suspension of the cells in distilled water promotes sporulation indicates the depletion of nutrients as the determining factor. When food is available, endospores are not formed, and those which had been formed under a local shortage of food soon germinate or tend to do so, even when conditions are not suitable for further growth. Experiments performed in connection with another problem show that in a medium containing a fermentable sugar but devoid of buffering value, endospores of this strain of *Bacillus mycoides* tend to germinate and perish because the low pH does not allow further growth. Cultures are obtained only when the medium is buffered. In distilled water and in incomplete media (for instance without a source of energy), the tendency to germinate is practically nil.

In drawing this obvious conclusion, we do not wish to be accused of teleologic tendencies which are detrimental to a final solution of the mechanism of sporulation. To say that cells starved in the presence of oxygen proceed to form endospores is to acknowledge a biological fact. Why the depletion of nutrients induces the cell to sporulate is a question yet to be answered. At the present, one can merely speculate.

#### SUMMARY

This paper is an investigation of some of the environmental factors which control the formation of endospores by a strain (C<sub>2</sub>) of *Bacillus mycoides*. A new technique of investigation is described.

It is shown that accumulation of the by-products of metabolism, such as the acid products of glycolysis, tends to inhibit sporulation. Oxygen may be necessary for some process in the formation of endospores, but its principal effect is in greatly increasing the rate of metabolism and the degree to which nutrients are utilized. As a result, there is a minimum accumulation of by-products; for instance, glucose is oxidized to CO<sub>2</sub> and probably water, and acids do not accumulate in the medium.

Although endospores may be formed within a long interval of pH, there is a definite optimum between pH 6.6 and 6.8. The significance of this optimum is not clear, and may well be related to the utilization of nutrients and the accumulation of by-products.

Vitamin B<sub>1</sub> promotes sporulation (but does not seem to affect growth) on agar slants of a simple medium in which there is a tendency for acid by-products to accumulate; it has no effect on either growth or sporulation in agar slants of vitamin-free casein hydrolyzate in which no acid by-products accumulate. The effect of the vitamin in the synthetic medium is indirect and due to an increase in the rate of decarboxylation of the acid by-products. It is assumed that the vitamin is readily synthesized in the casein hydrolyzate medium.

Gradual drying does not usually affect sporulation unless it affects growth and the availability of nutrients. With rare exceptions, when drying exerts an effect it is detrimental.

Suspending vegetative cells in distilled water promotes sporulation of the uninjured cells.

MgSO<sub>4</sub> increases the vegetative population. It has a beneficial effect on sporulation when the oxygen supply is low or limited.

Endospores do not germinate in distilled water or in an incomplete medium, such as a synthetic medium without a source of available energy, but they readily germinate in the presence of the necessary nutrients, even if the environment is unsuitable for further growth.

It is concluded that, in this strain of *Bacillus mycoides*, endospores are formed most readily by healthy cells faced with starvation in the presence of oxygen.

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# AMINO ACID REQUIREMENTS OF ACETOBACTER SUBOXYDANS

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*Acetobacter suboxydans* is of considerable interest because of its remarkable inability to dissimilate organic carbon compounds beyond the initial stages of oxidation (Visser't Hooft, 1925). This property of *A. suboxydans* and the fact that large amounts of substrate are transformed have suggested use of the organism for the large-scale production of various organic compounds. The oxidation of sorbitol to sorbose, which is used in the synthesis of vitamin C, is a well-established commercial process.

It has been necessary to add yeast extract or other similar organic nitrogenous complexes to media for *A. suboxydans*, as a source of unknown growth factors and nitrogen. Recently Underkofler, Bantz, and Peterson (1943) established that the required growth factors are pantothenic, nicotinic, and *p*-aminobenzoic acids. This has made it possible to determine for the first time the specific nitrogen requirements of *A. suboxydans*.

## METHODS

The strain of *A. suboxydans* was obtained from the University of Wisconsin. Stock cultures were maintained on yeast extract glycerol agar slants. The inoculum was prepared as described by Underkofler *et al.* (1943) except that the cells were washed once with water. One drop (ca. 0.05 ml) was used to inoculate 10 ml of medium contained in a 50-ml Erlenmeyer flask. Cultures were incubated at 30 C for 48 hours unless otherwise indicated. Growth was measured in an Evelyn photoelectric colorimeter.

The basal medium consisting of glycerol, hydrolyzed casein as nitrogen source, inorganic salts, and growth factors was that described by Underkofler *et al.* (1943) except that when amino acids,  $(\text{NH}_4)_2\text{SO}_4$ , or yeast extract was used as a source of nitrogen in place of hydrolyzed casein, tryptophane and cystine were omitted. All media were adjusted to pH 6.0.

The 20 amino acids (Merck) employed included synthetic leucine, isoleucine, valine, methionine, phenylalanine, glutamic acid, threonine, alanine, aspartic acid, lysine, serine, norleucine, and glycine. Those isolated from natural sources were cystine, tryptophane, tyrosine, arginine, histidine, proline, and hydroxyproline. Each 10 ml of medium received 2 mg of the natural amino acids and 4 mg of the synthetic amino acids. A larger amount of the latter was used since it was considered probable that only one of the two isomers would be available to *A. suboxydans*.

## EXPERIMENTS

In agreement with the results of Underkofler *et al.* (1943) a mixture of the 20 amino acids listed above could replace hydrolyzed casein for growth of *A.*

*suboxydans*. To determine which amino acids of the 20 are required, each one was omitted in turn from the medium and the effect on growth noted. Valine is essential since no growth occurred in its absence (table 1). Growth was slight without isoleucine or alanine. The need for isoleucine may be absolute since the small amount of growth obtained in its absence may be due to a small amount of isoleucine present as an impurity in the synthetic leucine of the medium (Hegsted and Wardell, 1944). Somewhat less than maximum development occurred when histidine, cystine, glutamic acid, aspartic acid, proline, or hydroxyproline was omitted. These amino acids can be classified as stimulatory. The remaining amino acids are not required by *A. suboxydans* since maximum growth occurred without them. On repeating this experiment, similar results were obtained except that cystine and aspartic acid, in contrast to their previous slight stimulatory action, had no effect on growth.

TABLE 1

*Effect of omission of individual amino acids from the "20 amino acid medium" upon the growth of Acetobacter suboxydans*

ESSENTIAL	PROBABLY ESSENTIAL	STIMULATORY	NONESSENTIAL
Valine.... . 98*	Isoleucine. . . 68 Alanine. . . 72	Histidine 45 Cystine . . . 37 Glutamic acid. . 41 Aspartic acid . . 38 Proline . . . 39 Hydroxyproline .. 38	Leucine . . . . . 31 Methionine 32 Tryptophane . 30 Tyrosine. . . 33 Phenylalanine.. 28 Threonine..... 30 Lysine. . . 33 Arginine. . . . 32 Serine . . . 28 Norleucine. . . 29 Glveine . . . 33

\* Per cent transmissible light of cultures grown without the amino acid indicated; uninoculated medium = 100. Cultures grown with all 20 amino acids gave a reading of 32.

Although valine, isoleucine, and alanine are essential components of the nitrogen requirements of *A. suboxydans*, growth did not take place in a medium containing only those three amino acids. This was not due to an insufficiency of nitrogen since a threefold increase of each amino acid did not alter the results. The further addition of histidine permitted slight growth, which increased on continued incubation as described below. The same effect was obtained also with proline but not with any of the other amino acids. Glutamic acid, aspartic acid, or hydroxyproline, when added to the four amino acids, did not materially increase growth. However, on the addition of either of the sulphur-containing amino acids, cystine and methionine, to the medium containing valine, isoleucine, alanine, and histidine, *A. suboxydans* developed fairly well although less than with the 20 amino acids. This marked effect of cystine and methionine is in sharp contrast to their inactivity in the previous experiment in which each amino acid, in turn, was omitted from the 20 amino acid mixture (table 1). In that

experiment, methionine was present in the medium when cystine was omitted and vice versa, so that the effect of each was masked by the presence of the other. The apparent nonessentiality of a particular amino acid in this type of experiment may, therefore, simply mean that some other remaining amino acid may serve the same but, nevertheless, necessary physiological function. On the contrary, absence of growth when one amino acid of the mixture is omitted is fairly conclusive evidence that the omitted amino acid is required for growth. Growth with the 20 amino acids was about equal to that obtained with hydrolyzed casein.<sup>1</sup>

The cultures in the medium containing valine, isoleucine, alanine, and histidine read only 94 after 48 hours of incubation, and this decreased to 78 on incubation for 3 additional days. Although growth was slow, sparse, and occurred in small clumps, all indicative of an unfavorable environment, *A. suboxydans* was successfully subcultured through six serial transfers in the same medium.

TABLE 2  
*Growth of Acetobacter suboxydans with various combinations of amino acids*

AMINO ACIDS IN BASAL MEDIUM	% TRANSMISSIBLE LIGHT	
	Exp. 1	Exp. 2
Valine + isoleucine + alanine + histidine + cystine (A)...	52	50
(A) + proline ...	29	34
(A) + hydroxyproline....	38	37
(A) + serine...	32	40
(A) + phenylalanine ..	41	37
20 Amino acids..	29	29
Hydrolyzed casein .	17	27

The combination of valine, isoleucine, alanine, and histidine represents, therefore, the smallest number of amino acids which can consistently support some growth of *A. suboxydans*.

Growth was raised to the same level as with 20 amino acids by the addition of proline to the medium containing valine, isoleucine, alanine, histidine, and cystine (table 2). Hydroxyproline, serine, and phenylalanine were only slightly less effective than proline. The somewhat limited specificity of the latter suggests that it may be utilized by *A. suboxydans* for the synthesis of the 15 or more other amino acids normally present in cellular proteins rather than directly as a structural building block. The remaining amino acids were substantially inactive when substituted for proline. It is thus established that the nitrogen requirements of *A. suboxydans* are satisfied by a mixture of six amino acids, namely, valine, isoleucine, alanine, histidine, cystine, and proline.

The medium containing these six amino acids, which supported, on repeated serial subculture, as much growth as the medium containing 20 amino acids, was used in the following experiments.

<sup>1</sup> Prepared from S. M. A. Corp. "vitamin-free" casein by hydrolysis with H<sub>2</sub>SO<sub>4</sub>.



*Effect of amino acid concentration and ammonium sulfate.* Increasing the concentration of the six amino acids in the basal medium from the customary 2 mg of the naturally occurring isomer per 10 ml of medium to 2, 3, or 4 times this amount did not appreciably increase the amount of cell substance. However, use of less than 2 mg caused a reduction in growth (table 3). With 0.01 to 0.1 mg of each amino acid, little or no growth occurred. The decrease may be due to a critical reduction of one or more of the essential amino acids, but probably not all. Nitrogen supply was a limiting factor under these conditions since addition of  $(\text{NH}_4)_2\text{SO}_4$  led to better growth. The effect of  $(\text{NH}_4)_2\text{SO}_4$  was decisive at the lowest amino acid concentrations, which alone failed to support development of *A. suboxydans*. At these low concentrations it was necessary to extend the incubation period to 4 days to obtain maximum effect of  $(\text{NH}_4)_2\text{SO}_4$ . There is no doubt, therefore, that *A. suboxydans* can utilize  $\text{NH}_4$ -nitrogen for growth provided it is supplied with the 6 amino acids which it is unable to synthesize or which are formed too slowly for normal development.

TABLE 3

*Influence of amino acid concentration and  $(\text{NH}_4)_2\text{SO}_4$  on the development of Acetobacter suboxydans in the "6 amino acid medium"*

AMOUNT OF EACH AMINO ACID PER 10 ML OF MEDIUM	GROWTH AFTER 2 DAYS		GROWTH AFTER 4 DAYS	
	Without $(\text{NH}_4)_2\text{SO}_4$	With $(\text{NH}_4)_2\text{SO}_4$	Without $(\text{NH}_4)_2\text{SO}_4$	With $(\text{NH}_4)_2\text{SO}_4$
mg*	<i>Per cent transmissible light</i>			
2.0	33	24	25	21
1.0	43	30	36	27
0.5	51	40	47	36
0.1	91	81	87	65
0.05	94	90	93	62
0.01	94	93	96	60

\* In terms of the *l*-isomer.

$(\text{NH}_4)_2\text{SO}_4$  could not be substituted for any of the 6 amino acids required by *A. suboxydans*.

*Influence of nitrogen source on rate and type of growth.* When bacto-yeast extract (0.4 per cent) was used as a source of nitrogen, *A. suboxydans* multiplied more rapidly and somewhat more extensively than with the six amino acids (figure 1). The lag phase with the latter lasted for about 12 hours compared to 6 hours with yeast extract. It was not reduced by the addition of asparagine, glutamine, glucose, or  $(\text{NH}_4)_2\text{SO}_4$ , although a combination of 0.1 per cent of the latter two substances increased total growth slightly. Also, substitution of mannitol or sorbitol for glycerol, addition of reducing agents (sodium thioglycollate and ascorbic acid), or use of inoculum grown in the amino acid medium did not affect either the rate or amount of growth in the amino acid medium. Delayed growth was noted both in stationary and agitated cultures. During the period of rapid increase, however, the rates in both media were approximately equal.

Growth with yeast extract occurred uniformly throughout the medium, and within 48 hours usually a delicate, fragile, surface membrane was evident. In contrast, initial growth in the synthetic amino acid medium adhered to the bottom and sides of the flask, leaving the body of the medium almost entirely clear. Later, a well-defined, surface pellicle formed, which could be dispersed only by vigorous shaking. The pellicle contained most of the cells of the culture. This type of surface growth was almost always obtained and appears to be characteristic for the synthetic amino acid medium.

*Deamination of amino acids by resting cell suspensions.* Cells were obtained from approximately 20-hour cultures grown on yeast extract glycerol agar medium. They were washed and suspended in M/15 phosphate buffer at pH

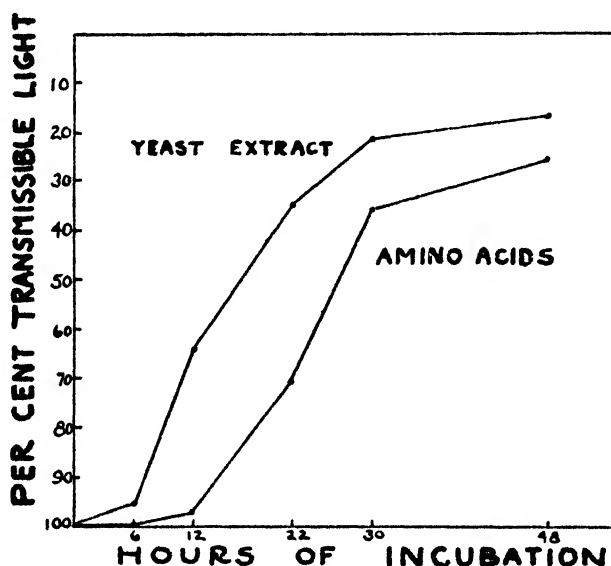


FIG. 1. RATE OF GROWTH OF *A. suboxydans* IN SYNTHETIC AMINO ACID AND YEAST EXTRACT MEDIA

6.7 to give a reading of 3 on the photometer. Five ml of cell suspension (equivalent to 32 mg of dry cells) and 5 ml of buffer containing an amount of amino acid equivalent to 2 mg of nitrogen of the natural isomer were mixed in a large tube fitted for aeration. One-tenth ml of tributyl citrate was added to reduce foaming, and a slow stream of either air or nitrogen was bubbled through the mixture (placed at 37°C) for 16 hours. The ammonia liberated was determined by distillation into N/70 HCl and titration of excess acid with N/70 NaOH.

Under aerobic conditions, 10 of the amino acids were deaminated either completely or more than 50 per cent (table 4). More than 100 per cent of the nitrogen in alanine, aspartic acid, and serine, calculated in terms of the *l*-isomer, was obtained as  $\text{NH}_3\text{-N}$ , indicating that both enantiomorphs of those amino acids were attacked. *Proteus* and *Pseudomonas aeruginosa*, under similar conditions, also decompose both isomers of alanine and serine (Bernheim, Bernheim, and

Webster, 1935; Webster and Bernheim, 1936). Seven of the amino acids were deaminated to the extent of 50 per cent or less. Little or no  $\text{NH}_3$  was obtained from leucine, isoleucine, or valine. Since the latter two amino acids also are required, preformed, for growth of *A. suboxydans* it is probable that they are utilized for synthesis of protein without extensive modification.

Anaerobically, little or no deamination occurred with any of the amino acids with the exception of serine both isomers of which were attacked.

According to these results, *A. suboxydans* decomposes amino acids primarily by a process of oxidative deamination. This is in contrast to the data of Miyaji (1925) which indicate that other acetic acid bacteria reductively deaminate glycine and tyrosine, but is perhaps more in accord with the highly aerobic character of these organisms.

TABLE 4

*Aerobic deamination of amino acids by resting cell suspensions of Acetobacter suboxydans*

PER CENT NITROGEN LIBERATED AS $\text{NH}_3$		
100-50	50-10	10-0
Glutamic acid	Cystine	Leucine
Aspartic acid	Methionine	Isoleucine
Alanine	Tyrosine	Valine
Lysine	Phenylalanine	
Arginine	Threonine	
Tryptophane	Norleucine	
Histidine	Glycine	
Serine		
Proline		
Hydroxyproline		

## SUMMARY

The amino acids required by *Acetobacter suboxydans* for growth were determined. The organism multiplies to a limited extent in a medium consisting of glycerol, salts, essential growth factors, and a combination of valine, isoleucine, alanine, and histidine as nitrogen source. Growth is considerably improved by the addition of either cystine or methionine. The further addition of proline increases growth to the level obtainable with a mixture of 20 amino acids or hydrolyzed casein. However, development is not so rapid and somewhat less extensive than with yeast extract. At suboptimum concentrations of the six required amino acids,  $(\text{NH}_4)_2\text{SO}_4$  stimulated growth.

Resting cell suspensions of *A. suboxydans* deaminate most amino acids under aerobic conditions. Both optical isomers of alanine, serine, and aspartic acid are attacked. Under anaerobic conditions only serine is deaminated to any appreciable extent.

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# CLOSTRIDIUM PARASPOROGENES, AN INVALID SPECIES

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Twenty-five years ago McIntosh (1917) isolated an organism from a gas gangrenous wound which gave the biochemical reactions of *Clostridium sporogenes*, but whereas the typical *C. sporogenes* colonies are fimbriate, frequently with wide-spreading rhizoids, the new organism grew in almost entire, smooth, low, convex colonies. This was first described as *Bacillus* type XII (McIntosh, 1917) and later as *Bacillus parasporogenes* (McIntosh, 1919). It is included in the first and subsequent editions of *Bergey's Manual* as *Clostridium parasporogenes* (McIntosh) Bergey *et al.* No attention appears to have been given the organism since that time, but Weinberg *et al.* (1937) suggest that "il s'agit probablement d'une race de *B. sporogenes*." This is quoted by Spray (*Bergey's Manual*, 5th edition).

Only two cultures, which appear to be direct descendants of McIntosh's original culture could be located: no. 693 of the American Type Culture Collection, and one kindly supplied by Dr. McCoy, University of Wisconsin, labeled *C. parasporogenes*, strain McIntosh. The colony structure of the two cultures has been compared in some detail with nine cultures from various sources labeled *C. sporogenes*. At the same time a comparison has been made of variation in the two sets of cultures.

All these cultures, both those labeled *C. parasporogenes* and *C. sporogenes*, gave typical biochemical reactions for *C. sporogenes* (Reed and Orr, 1941).

"*C. parasporogenes*" no. 693, A. T. C. Collection, the stock culture, on plating on blood agar, peptone thioglycollate agar, or subsurface plates on semisolid peptone thioglycollate agar (Reed and Orr, 1941), developed about equal numbers of smooth and rough colonies. The smooth colonies were nearly entire, smooth, flat to low convex on the surface of solid media and lenticular to spherical smooth in poured plates of semisolid medium, i.e., they fitted exactly McIntosh's original description of *C. parasporogenes* (figure 1). The rough colonies, in sharp contrast, were spreading fimbriate colonies with irregular rhizoids on the surface of solid media and definitely woolly in subsurface growth, i.e., typical *C. sporogenes* colonies (figure 2).

Rough and smooth colonies were fished from primary plates made from the original stock culture. After eight generations of single colony selection the rough strain still produced only rough colonies. The most characteristic smooth colonies were selected, and single colony isolations carried out for eight generations. The majority of colonies in all these cultures were smooth, but a few rough colonies continued to appear, and selected rough colonies always bred rough. This is clearly a case of variation from the *C. parasporogenes* smooth type of colony to the *C. sporogenes* rough type.

"*C. parasporogenes*, strain McIntosh," Dr. McCoy's collection, when plated from the original culture and after repeated transfers in various media, yielded only the characteristic fimbriate to rhizoid colonies characteristic of *C. sporogenes*. Repeated selection of the least fimbriated colonies failed to establish a smooth type. If this is a descendant of McIntosh's original culture, it is apparent that variation from smooth to a relatively stable rough form has occurred.



1



2

FIG. 1. COLONY FORM *C. PARASPOROGENES*FIG. 2. COLONY FORM *C. SPOROGENES*

*C. sporogenes* no. 11056, A. T. C. Collection, on repeated plating also produced only characteristic rough colonies, but after selecting the least rhizoid colonies for some ten generations a few perfectly smooth colonies appeared. Repeated single colony transfers resulted in a true breeding smooth form which remained smooth for several generations. But after aging for a month in chopped meat the strain had partly reverted to the rough form. By selection, therefore, a typical *C. sporogenes* becomes, for a time, a *C. parasporogenes* type.

*C. sporogenes* no. 7689, A. T. C. Collection, produced on the first plating both smooth and rough colonies. The smooth colonies, resembling *C. parasporogenes*, bred true to type for several generations of single colony selection. Aging for a month in chopped meat failed to induce any change. But four daily transfers in broth containing 1 per cent LiCl resulted in a 50 per cent reversion to the rough type. This rough type bred true for several generations as a characteristic *C. sporogenes*.

Six additional cultures of *C. sporogenes* yielded only typical rough colonies with fimbriate to rhizoid margins. Daily or twice daily transfers in fluid media for long periods failed to produce any detectable variation in colony structure.

Weinberg *et al.* (1937) state that there is an antigenic difference between *C. sporogenes* and *C. parasporogenes*. Smooth and rough forms isolated from *C. parasporogenes*, strain no. 693, as described in an earlier paragraph, have been compared in agglutination reactions. Living 24-hour cultures of the smooth and the rough forms were injected into rabbits intravenously. Five doses were given at 4-day intervals, and the animals were bled 10 days after the last dose. Agglutination reactions were carried out with these sera and

TABLE 1  
*Agglutination of smooth and rough types by antismooth and antirough sera*

ANTIGEN	ANTISERUM	MAXIMUM AGGLUTINATION TITER
Smooth type	Antismooth	1-80
Rough type	Antismooth	1-20
Smooth type	Antirough	1-2
Rough type	Antirough	1-40

similar living cultures of smooth and rough forms as antigens. Results are summarized in table 1. This clearly indicates an antigenic difference; the difference, however, is comparable with what is observed in S and R forms of many species.

Since colony structure and antigenic content are the only differences which have been indicated between *C. sporogenes* and *C. parasporogenes* and since *C. sporogenes* has been shown to produce, by variation, the *C. parasporogenes* form, it is suggested that *C. parasporogenes* is an invalid species.

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# STORAGE OF INFLUENZA VIRUS FOR USE IN TYPING CLINICAL CASES

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The practicability of typing cases of influenza at hospital laboratories in the event of a future outbreak was suggested in an earlier report from this section (Florman and Crawford, 1944). At that time a simple modification of the Hirst technique was presented. For its application the only reagents which might not be readily available in clinical laboratories would be viruses of satisfactory hemagglutinating titers. These might be furnished by some large central laboratory. In order to determine the most satisfactory manner in which these hemagglutinating reagents might be supplied, the present study was undertaken.

## PLAN OF STUDY

Allantoic fluids containing influenza A(PR-8) and B(Lee) viruses were diluted with buffered saline and various bacteriostatic agents, and examined for hemagglutinating titers at regular intervals over a period of 9 weeks. The agents tested were 0.2 per cent formaldehyde (0.07N), 0.2 per cent sulfanilamide, 33 per cent buffered glycerol, and, as a control, a phosphate-buffered 0.9 per cent solution of sodium chloride of pH 7.6. Two-ml portions of each were prepared in duplicate and stored in small, sterile, screw-top bottles. Each batch was adjusted to give a hemagglutinating titer with chicken cells of 1-320. One set was kept at room temperature (approximately 20 C) and the other in the icebox (approximately 4 C). At regular intervals some of the material from each bottle was tested for its hemagglutinating capacity, bacterial contamination, and infectivity.

## RESULTS

The hemagglutination titers are given in table 1. The slight differences in titer from week to week are attributed to technical variations. The only unsatisfactory means of storage was with the 0.2 per cent formaldehyde. All others maintained a satisfactory titer for at least 61 days. At the end of this period they were used successfully with a known influenza serum. The fluids stored in the icebox tended to keep slightly better, though this difference was not striking.

Bacteriological cultures on blood agar were made from each of the bottles at the start of the experiment, after one month, and again after two months. No special precautions were taken during the frequent openings of the bottles in order to simulate conditions which might prevail in the field. At the end of

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one month, except for the formaldehyde-treated material, all fluids stored at room temperature were contaminated. At the end of this same period, with the exception of slight growth in one of those treated with glycerol, no bacteria were cultured from any of the fluids stored at 4 C. After 61 days of icebox storage both of the fluids to which formaldehyde and sulfanilamide had been added and one of the saline controls were bacteria-free.

Tests for infectivity were performed by inoculating the allantoic cavities of 11-day embryonated chick eggs. After inoculation these eggs were incubated for 48 hours more at 37 C and then stored overnight at 4 C. The allantoic fluids were then harvested and tested for hemagglutinating titer. The first inoculations were made after two weeks. By this time all of the fluids stored at room temperature had lost their infectivity. However, after 21 days the influenza A saline control which had been kept at 4 C showed a titer of 1-320

TABLE 1

*Serial hemagglutinating titers after storage of allantoic fluids containing influenza virus*

DAYS OF STORAGE	AT ROOM TEMPERATURE (APPROX. 20 c)								AT ICEBOX TEMPERATURE (APPROX. 4 c)							
	Influenza A				Influenza B				Influenza A				Influenza B			
	0.2% form.	0.2% sulfa.	33% gly.	0.9% sal.	0.2% form.	0.2% sulfa.	33% gly.	0.9% sal.	0.2% form.	0.2% sulfa.	33% gly.	0.9% sal.	0.2% form.	0.2% sulfa.	33% gly.	0.9% sal.
0	320	320	320	320	320	320	320	320	320	320	320	320	320	320	320	320
7	<10	160	320	320	<10	320	320	320	40	320	160	320	40	640	320	320
14	<10	320	320	320	<10	320	160	160	<10	160	320	320	<10	320	320	320
21	10	320	320	160	<10	640	320	160	<10	320	320	320	<10	320	320	640
27	<10	320	320	320	<10	640	320	160	<10	320	160	320	<10	160	160	320
34	<10	640	<10	640	<10	640	320	320	<10	320	320	160	20	640	320	640
41	<10	320	160	160	<10	320	160	320	<10	320	160	320	<10	320	320	320
48	<10	320	160	320	<10	320	320	320	<10	320	160	160	<10	320	160	320
56	<10	320	160	160	<10	320	320	320	10	320	160	160	<10	640	320	320
61	<10	160	160	160	<10	640	320	320	<10	320	320	320	<10	640	320	160

in one of three eggs. When retested after 61 days, there was no evidence of infectivity. In contrast, two of three eggs inoculated with the 0.2 per cent sulfanilamide-treated fluid and stored in the ice box showed a titer of 1-320 after four weeks. At the end of the experiment, 61 days, there were sufficient viability and multiplication to give a titer of 1-640 in one of three eggs inoculated with this same fluid.

All of the fluids developed some turbidity on storage. This was slightly more marked in those kept at room temperature. However, in no instance did this interfere with the performance of the test.

## COMMENT

As part of an earlier experiment, some influenza A and B virus, adjusted to give a hemagglutinating titer of 1-320, was stored in the icebox in 33 per cent glycerol and in buffered saline. After 139 days a titer of at least 1-160 could

be demonstrated with each batch of virus. Somewhat similar observations have been made by others with influenza A (Pearson, 1944).

It is noteworthy that the presence of contaminating bacteria did not impair the usefulness of these fluids in the hemagglutinating reaction. Nevertheless reasonable precautions against contamination should be taken since certain bacteria, e.g., strains of *Escherichia coli*, possess the property of agglutinating red cells (Rosenthal, 1943).

Formaldehyde in the strength used in these experiments (0.2 per cent) is an excellent bacteriostatic agent, but over a short period of time it destroys the virus. This effect has been attributed to its action on the virus protein (Knight and Stanley, 1944). Glycerol (33 per cent) and sulfanilamide (0.2 per cent) have no untoward effects.

Pearson (1944) recently reported that the hemagglutinating activity of influenza A (PR-8) was no better conserved in frozen and dried allantoic fluid than in material stored in the fluid state at the same temperature.

The present experiment demonstrates again that the infective and hemagglutinating capacities of influenza virus are separable.

#### CONCLUSIONS

No special storage precautions are necessary for the maintenance of a satisfactory hemagglutinating agent for typing influenzal sera for a period of two months.

However, since slightly better results are obtained with their use, 0.2 per cent sulfanilamide and icebox storage are recommended.

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*Addendum:* Since this report was submitted for publication, an exhaustive study by Stanley of the preservation of purified influenza virus has appeared (Stanley, W. M. 1945. The preparation and properties of influenza virus vaccines concentrated and purified by differential centrifugation. *J. Exptl. Med.*, **81**, 193-218). His findings with the untreated highly concentrated and purified virus were of a similar nature to the observations reported here for crude virus-containing allantoic fluids.



## NOTES

### A NEW TYPE SALMONELLA

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The two cultures studied were isolated from American soldiers acting as food handlers in a replacement depot somewhere in North Africa. Both cultures possessed the cultural and biochemical characteristics of *Salmonella*, with the exception of gelatin liquefaction. Acid and gas were produced from arabinose, dulcitol, glucose, inositol, and xylose. Lactose, salicin, and sucrose were not fermented. Dextro-tartrate and citrate were attacked, hydrogen sulfide was produced, but no indole was formed, and gelatin was liquified within 10 to 15 days. The proposed name is *Salmonella canastel*.

Alcohol-treated suspensions of the microorganism were agglutinated to the titer of *Salmonella gallinarum* O serum (IX, XII. . .). The presence of antigen I was not detected. In absorption tests the bacterium removed all agglutinins from *S. gallinarum* O serum. The O antigens of *Salmonella canastel* are IX, XII. . . .

The organism was diphasic and phase 1 was agglutinated to titer by *Salmonella tennessee* (z<sub>29</sub>) antiserum. In absorption tests it was found that *S. canastel* reduced the titer of z<sub>29</sub> antiserum from 10,000 to 200. For diagnostic purposes phase 1 of the microorganism may be designated as z<sub>29</sub>.

Phase 2 of *S. canastel* was agglutinated by serums derived from all the non-specific phases of the Kauffmann-White classification. When tested with absorbed serums containing agglutinins for factors 2, 3, 5, 6, 7, and 10, respectively, it was agglutinated only by 3 and 5 serums. In absorption tests, *S. canastel* reduced the agglutinins of *Salmonella cholerae-suis* var. *kunzendorf* antiserum from a titer of 10,000 to a titer of 200. Phase 2 of *S. canastel* may be expressed as 1, 3, 5. . . .

#### SUMMARY

The diagnostic formula of *Salmonella canastel* is IX, XII. . . : z<sub>29</sub>-1, 3, 5. . . . This is the first time the antigenic factor z<sub>29</sub> has been found in a diphasic type. It is noteworthy that *Salmonella dar-es-salaam* (IX, XII. . . : 1, w. . . -e, n. . .), a member of somatic group D, also liquefies gelatin.

### A NOTE ON THE TRIBE MIMEAE (DE BORD)

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During the past several months 19 cultures were isolated and classified in the three genera of the tribe *Mimeae* described in 1942 by De Bord (Iowa State

Coll. J. Sci., 16, 471). Eight cultures which failed to ferment carbohydrates were classified in the genus *Mima*, whereas 10 cultures which produced acid were listed in *Herellea*, and 1 culture which produced acid and gas was placed in the genus *Colloides*. They were recovered from previously unreported sources, and show certain variances in biochemical features from the described species, certain pathogenicity relationships, and serological types within a species.

Morphologically all cultures agreed with the original descriptions in showing varying degrees of pleomorphism. The outstanding form was a gram-negative diplococcus not unlike members of the genus *Neisseria* (De Bord: J. Lab. Clin. Med., 28, 710). In all cases, rods, filaments, or both, as well as diplococci, could be demonstrated. All of the cultures showed gram stain variability although most of the organisms in any preparation gave a negative reaction.

Three strains in the genus *Mima* were recovered from therapy-resistant gonorrhea in the male, 3 from war wounds, 1 from a chaneroid lesion, and 1 from brain tissue following head injury. Five strains agreed with the description of the type species or its "oxidase positive" variant. Three variant related strains failed to agree with the type species; 1 culture utilized citrate, and 2 reduced nitrates to nitrites.

Guinea pigs were employed for pathogenic studies. Intraperitoneal and subcutaneous inoculations were made with 0.5 ml of a 24-hour broth culture.

One strain in the genus *Mima* proved lethal to the guinea pig within 12 hours when injected intraperitoneally. Autopsy findings showed definite inflammation of the adrenal glands and the heart's blood contained the injected organisms. Subcutaneous inoculation failed to produce either lesions or death. This pathogenic culture was recovered from a war wound and agreed with the "oxidase positive" variant.

Nine of the 10 cultures classified as *Herellea* were recovered from cerebrospinal fluid following head injury, and 1 from urine following an operation for ureteral stricture. All cultures were physiologically identical but failed to agree with the description of the type species by fermenting lactose and failing to ferment either mannitol or dulcitol. Serologically they were separated into 3 distinct types by means of the "Quellung" reaction. Intraperitoneal injection of all *Herellea* cultures killed guinea pigs in 12 to 42 hours. Subcutaneous inoculations proved nonpathogenic, and autopsy findings were the same as those described for the genus *Mima*.

A single culture recovered from a clinical case of "gas gangrene" was placed in the genus *Colloides*. It did not agree with the description of the type species since it failed to produce indole and was methyl-red-negative. This strain killed guinea pigs in 18 hours following intraperitoneal injection. It was non-pathogenic by subcutaneous inoculation. Autopsy findings were the same as those already described.

Extensive work is in progress dealing with pathogenicity, specific antigenic capsular substances, and generic antigen relationships.

## A PARACOLONLIKE BACILLUS ISOLATED FROM COLITIS IN AN INFANT<sup>1</sup>

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In previous communications a group of paracolon bacilli which are definitely pathogenic for animals was described (J. Infectious Diseases, **70**, 185; **73**, 229). These strains are similar to "*Salmonella arizona*" of Kauffmann and they attack both warm- and cold-blooded animals. Their occurrence in snakes was recently recorded by Hinshaw and McNeill (Cornell Vet., **34**, 248). The group is closely related serologically to *Salmonella*, some strains having complete *Salmonella* O antigens as well as H antigens which are similar to those of certain *Salmonella* strains. They differ from *Salmonella* in that they liquefy gelatin slowly and ferment lactose. The fermentation of lactose varies in different strains from those that produce acid and gas in 24 hours to those that must be transferred serially in lactose in order to demonstrate a slight production of acid from the sugar.

The purpose of the present note is to record the occurrence of a member of this group in an intestinal infection in man. In September, 1943, a culture was received from the laboratories of the Virginia State Department of Health for identification. It was isolated from the stools of an eleven-month-old baby affected with acute colitis. No other pathogenic organism was found in the specimen. The organism was a methyl-red-positive, Voges-Proskauer-negative strain which produced large amounts of hydrogen sulphide, but did not form indole. Gelatin was liquefied after 20 days' incubation. Growth occurred on Simmons' citrate agar and in Koser's citrate medium. Dextro, levo, and meso tartrate were not attacked, but mucate was utilized. Acid and gas were produced from glucose, maltose, rhamnose, xylose, arabinose, sorbitol, and mannitol. Lactose, sucrose, inositol, dulcitol, adonitol, and salicin were not fermented.

Serological examination revealed that the culture possessed antigens typical of the "*S. arizona*" group. The H antigens were identical with those of "arizona" whereas the O antigens were identical with those of type 3 paracolon of Edwards, Cherry, and Bruner (*loc. cit.*). Inasmuch as the culture was a typical member of the "arizona" group as judged by its antigenic composition, it has been considered a paracolon bacillus even though it has been impossible to demonstrate lactose fermentation by this strain. The culture was transferred serially in lactose broth for eight months and was also transferred serially in broth containing 5 per cent lactose. No evidence of fermentation was observed. The culture in question is one of those strains the occurrence of which renders difficult the establishment of sharply defined genera in the Enterobacteriaceae.

<sup>1</sup> The investigation reported in this paper was conducted in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.



*Acknowledgement.* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Kentucky Agricultural Experiment Station.

## A NEW SALMONELLA TYPE: SALMONELLA PAPUANA

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*Salmonella papuana* was isolated<sup>4</sup> from a stool specimen showing blood and mucus. No further clinical history is available. The specimen came from a patient in a U. S. Army Station Hospital located at Port Moresby in the province of Papua. The culture was received as a suspected *Salmonella* and had been submitted for definitive classification and antigenic analysis.

*Cultural characteristics.* The organism possesses the morphological, cultural, and biochemical characteristics of the *Salmonella* genus. It is a gram-negative, motile, nonsporeforming rod. Acid and gas are produced in glucose, maltose, mannitol, xylose, arabinose, dulcitol, rhamnose, and sorbitol. Lactose, sucrose, inositol, and salicin are not fermented. Dextro-tartrate and citrate are utilized. It produces hydrogen sulfide but does not form indole or liquefy gelatin.

*Serological study.* Alcohol-treated suspensions of the organism were agglutinated by *Salmonella oranienburg* O serum (VI, VII. . .) and by *Salmonella newport* O serum (VI, VIII. . .) which places the strain in group C according to the Kauffmann-White diagnostic schema. When tested with single factor absorbed serums, it was found to possess somatic antigens VI<sub>1</sub>, VI<sub>2</sub>, and VII. . .

Examination of the flagellar antigens of the new type revealed that it was diphasic. Phase 1 was flocculated to the titer of serum derived from phase 1 of *Salmonella rubislaw* (r) and in absorption tests *S. papuana* removed all flagellar agglutinins from *S. rubislaw* serum. Phase 1 of *S. papuana* is, therefore, designated as r.

Phase 2 of *S. papuana* was agglutinated by *Salmonella glostrup*, phase 2 (enz<sub>15</sub>. . .) and *Salmonella abortus-equi* (enx. . .) sera. Absorption tests showed that *S. papuana* removed all of the flagellar agglutinins from *S. glostrup*, phase 2 serum, but would not remove all of the agglutinins from *S. abortus-equi* serum. Further studies using absorbed single factor x. . . and z<sub>15</sub>. . . sera showed that phase 2 of *S. papuana* may be designated as enz<sub>15</sub>. . .

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<sup>4</sup> Isolated by First Lieutenant S. B. Goldwasser, Sn-C.

## SUMMARY

A new *Salmonella* type isolated from a human stool specimen has been described. No reference has been found in the literature to an organism possessing the same antigenic structure. The organism described is designated *Salmonella papuana* and has the antigenic formula VI<sub>1</sub>, VI<sub>2</sub>, VII...: r; enz<sub>15</sub>...

## PURIFICATION OF HIGH TITER TETANUS TOXIN

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Eaton and Gronau (J. Bact., **36**, 423) have purified tetanus toxin by (1) precipitation with cadmium chloride followed by elution of the toxin with 2 per cent sodium phosphate at pH 7.8, and (2) precipitation with ammonium sulfate followed by dialysis. Their crude toxin contained  $250 \times 10^{-6}$  mg N<sub>2</sub> per mouse M.L.D. whereas the purified preparations contained *ca.*  $2 \times 10^{-6}$  mg N<sub>2</sub> per M.L.D.

TABLE 1

Total nitrogen and M.L.D. in crude and purified tetanus toxin

TOXIN NO.	PURIFICATION PROCEDURE	TOTAL N <sub>2</sub>	M.L.D. PER ML	N <sub>2</sub> PER M.L.D.	L <sub>t</sub> PER M.L.D. $\times 10^{-4}$	YIELD*	PURIFICATION†
		mg/ml		mg		per cent	
1	crude NH <sub>4</sub>	2.36	800,000	$2.95 \times 10^{-6}$	0.9		
		0.0147	400,000	$0.037 \times 10^{-6}$		50	80
2	crude NH <sub>4</sub> Cd Cd, NH <sub>4</sub>	2.39	300,000	$7.97 \times 10^{-6}$	1.3		
		0.0166	200,000	$0.083 \times 10^{-6}$	1.8	67	96
		0.0180	200,000	$0.090 \times 10^{-6}$	1.0	67	89
		0.0057	100,000	$0.057 \times 10^{-6}$	1.3	33	140
3	crude Cd, NH <sub>4</sub>	2.36	400,000	$5.90 \times 10^{-6}$	1.1		
		0.0075	200,000	$0.038 \times 10^{-6}$	1.1	50	155
4	crude Cd, NH <sub>4</sub>	2.37	300,000	$7.90 \times 10^{-6}$	1.0		
		0.0035	150,000	$0.023 \times 10^{-6}$	1.2	50	344

$$* \text{Yield} = \frac{\text{M.L.D./ml (purified)}}{\text{M.L.D./ml (crude)}} \times 100.$$

$$\dagger \text{Purification} = \frac{\text{mg N}_2/\text{M.L.D. (crude)}}{\text{mg N}_2/\text{M.L.D. (purified)}}$$

A strain of *Clostridium tetani* is now available in this laboratory which is capable of producing remarkably high yields of toxin on certain types of media

containing an excess of iron (Mueller and Miller, J. Immunol., in press). For purposes of attempted purification it has been grown in a heart infusion medium containing glucose, tryptic digest of casein, and an excess of reduced iron. The crude toxins obtained from this medium contain 200,000 to 800,000 mouse M.L.D. per ml (ca.  $5 \times 10^{-6}$  mg N<sub>2</sub> per M.L.D.). Several lots of the crude toxin have been purified by precipitation with cadmium chloride according to Eaton and Gronau, by precipitation with 0.5 saturated ammonium sulfate followed by dialysis at 0 to 5 C against 0.9 per cent NaCl, or by precipitation with cadmium chloride and reprecipitation with ammonium sulfate. The best preparation (table 1, toxin no. 4) contained  $0.023 \times 10^{-6}$  mg N<sub>2</sub> per M.L.D. The relatively constant ratio, L<sub>t</sub>/M.L.D., indicated that losses during purification did not result from inactivation of toxin; rather, losses resulted largely from incomplete elution from the cadmium precipitate.

Although the N<sub>2</sub>/M.L.D. ratio represents a toxin of a hundredfold greater purity than any previously obtained, we have as yet no evidence that a limit of purification has been reached. Further, these data do not yet exclude the possibility that the toxic entity is nonprotein and that it is carried through the purification procedures by adsorption on inactive protein. Indirect evidence, however, argues against this assumption: 80 per cent of the total nitrogen of the purified preparations is precipitated by 5 per cent trichloroacetic acid and 70 per cent by 1 per cent metaphosphoric acid; and the total nitrogen, calculated as protein, constitutes  $100 \pm 2$  per cent of the dry weight in the purified preparations.

## TRIPLE-SUGAR IRON AGAR MEDIUM FOR THE IDENTIFICATION OF THE INTESTINAL GROUP OF BACTERIA

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Kligler's iron agar medium has replaced Russell's and Krumwiede's medium in many laboratories as a primary differential medium for enteric pathogens. The advantage of Kligler's medium over Russell's and Krumwiede's lies in the reaction for H<sub>2</sub>S. Since practically all members of the *Eberthella* and *Salmonella* genera produce H<sub>2</sub>S whereas those of the genus *Shigella* do not, this reaction is of great practical importance in combination with the characteristic differential reactions of acid and gas production first suggested by Russell, and by means of which enteric pathogens can be distinguished from coliform and other non-pathogenic bacteria. The value of Kligler's medium has been limited by the fact that H<sub>2</sub>S is not produced by all strains of *Eberthella typhosa* and by certain members of the genus *Salmonella*. Production of H<sub>2</sub>S is sometimes so slow that reactions may be negative at the important 18 to 24-hour incubation period and the reactions are not always so clear-cut as is desirable.

A new triple-sugar iron agar (TSI) medium has been developed which gives

more satisfactory reactions, that is, reactions which are more clear-cut for acid and gas, and more sensitive for  $H_2S$ . This is important since selection of carbohydrate test media to be used for preliminary identification of members of the *Eberthella*, *Salmonella*, or *Shigella* genera should be based on the TSI reactions. The formula for the TSI medium is given below.

To each liter of distilled water, add

Agar (dry).....	13.0 g
B.B.L. nutripeptone* . . . . .	20.0 g
Sodium chloride.....	5.0 g

Dissolve in the Arnold sterilizer or in flowing steam in the autoclave. When melted, adjust to pH 7.5.

Admix

Lactose... . . . .	10.0 g
Sucrose... . . . .	10.0 g
Glucose... . . . .	1.0 g
Sodium thiosulfate....	0.2 g
Ferrous ammonium sulfate...	0.2 g

Check the pH, which should then be 7.4.

Lastly, add

Phenol red (1% aqueous solution) . . . . .	2.5 ml.
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Dispense approximately 5 ml. in 15 x 100 mm tubes. Autoclave at 12 pounds pressure for 15 to 17 minutes.

\* Bacto-peptone 15 g plus bacto-proteose peptone 5 g gives equally good results.

Sodium sulfite and meat extract have not been found to increase materially the production of  $H_2S$ . The role of sucrose in the medium is to eliminate certain sucrose-fermenting, non-lactose-fermenting bacteria of the *Proteus* and paracolon groups.

All of the *Salmonella* cultures listed in Circular 54 of the University of Kentucky, plus 26 types recently furnished by Dr. P. R. Edwards, were used in this investigation. Very few *Salmonella* organisms failed to produce  $H_2S$ , notably *S. paratyphi* A, *S. abortus-equi*, *S. typhi-suis*, *S. berta*, *S. sendai*, and *S. papuana*. *S. cholerae-suis*, hitherto distinguishable from *S. cholerae-suis* var. *kunzendorf* by its failure to produce  $H_2S$ , produced it in the medium described here, though in lesser quantity than did the Kunzendorf variety. *Eberthella typhosa* and *S. gallinarum* are indistinguishable in this medium.

# SALMONELLA TYPES ISOLATED IN MARYLAND BETWEEN 1936 AND 1943

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Close identification of *Salmonella*-like bacteria has not been possible until the development by Kauffmann, by White, and by Edwards of a method of antigenic analysis. (For references, see Univ. Ky. Agr. Expt. Sta., Circ. 54.) During recent years all cultures routinely isolated and identified as belonging to the *Salmonella* group have been preserved by the lyophilic process. These cultures, therefore, afforded an opportunity to check the older method of identification and to give information on the types of *Salmonella* which had been found in Maryland.

TABLE 1  
*Identification and source of Salmonella cultures*

SALMONELLA TYPE	GROUP	SOURCE OF CULTURE		
		Feces	Blood	Other
<i>S. typhimurium</i> .....	B	20	2	pus 1
<i>S. paratyphi</i> B.....	B	15	6	
<i>S. derby</i> .....	B	6		
<i>S. montevideo</i> .....	C-1	5		
<i>S. oranienburg</i> .....	C-1		3	sp. fl. 1
<i>S. cholerae-suis</i> .....	C-1		2	
<i>S. newport</i> .....	C-2	3	1	
<i>S. oregon</i> .....	C-2	1		
<i>S. panama</i> .....	D	12	4	
<i>S. enteritidis</i> .....	D			G. P. spleen 1
<i>S. anatum</i> .....	E	1		
<i>S. poona</i> .....	Further	1		
<i>S. hwhittingfoss</i> .....	Further	1		
Total.....		65	18	3

Of 101 *Salmonella* cultures in the collection, 9 were found to be dead. Six were found to be paracolon organisms. The rest were *Salmonella* species according to the Kauffmann-White schema.

The identification of the 86 *Salmonella* strains and the sources from which they were obtained is given in table 1.

One strain of *Salmonella paratyphi* B was found to be anaerogenic. Altogether only 13 types of a group of nearly 150 known types were found.

# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## EASTERN PENNSYLVANIA CHAPTER

ONE HUNDRED AND SEVENTY-SEVENTH MEETING, PHILADELPHIA COUNTY MEDICAL  
SOCIETY BUILDING, PHILADELPHIA, PA., JANUARY 23, 1945.

**THE ACTION OF CERTAIN POORLY ABSORBED SULFONAMIDES ON THE INTESTINAL FLORA OF RATS.** *R. J. Strawinski, W. F. Verwey, and R. Munder*, Department of Bacteriology, Medical Research Division, Sharp and Dohme, Philadelphia, Pa.

**ANTIGENIC STRUCTURE AND SPECIFICITY OF LUMINOUS BACTERIA.** *George H. Warren*, Department of Bacteriology and Immunology, Jefferson Medical College, Philadelphia, Pa.

The agglutination, agglutinin absorption, and precipitation reactions of eight species of marine and "fresh water" luminous bacteria were studied.

Cross agglutination results indicate an agglutinogenic specificity for *Achromobacter fischeri*, *Photobacterium phosphoreum*, *Bacillus sepiae*, and *Bacillus pierantonii*. Cross reactions occur between *Achromobacter harveyi* and *Photobacterium splendidum*, between *Vibrio albensis* and *Vibrio phosphorescens*, and between *Photobacterium splendidum* and *Bacillus sepiae*. The *V. albensis* and *V. phosphorescens* cross agglutination expresses the only reciprocal relation.

Luminous cell filtrates reveal an ability to produce both specific and group agglutinins. This suggests that the agglutinogenic factors derived from luminous cells are all present in their filtrates, and points to the classification of the latter as "complete" antigens.

Cross precipitation experiments with both luminous-cell immune sera and filtrate immune sera exhibit marked group reactions. A distribution of common antigens or a possible dissociation of antigenic groups during the luminous-cell-filtrate transition is suggested.

A specific antigen-antibody reaction which interferes with the luminescent system has not been noted.

**ANTIGENIC VARIATION IN PURE CULTURES OF PARAMECIA.** *James A. Harrison and Elizabeth H. Fowler*, Department of Biology, Temple University, Philadelphia, Pa.

Antigenic variation was followed in many pure cultures of the ciliated, free-living protozoan *Paramecium aurelia*. Our attention was devoted largely to the characterization of many new cultures propagated from individual members of pedigreed populations which contained more than one antigenic type of cell. The results indicated that cultures developed quickly from single cells usually were more homogeneous antigenically than the parent cultures, and usually, though not always, were essentially similar in antigenic character to the individual cells from which they were propagated. Some of the variant cultures propagated from individual cells reverted in antigenic character to that of the parent cultures in the short period of 10 days; others remained stable in the variant phase for periods up to 18 months, then reverted suddenly to the character of the parent cultures. Several unsuccessful attempts were made to develop, in addition to the antigenically variant cultures propagated from cells selected at random, still other antigenic types of variants by growing the cultures in selected antisera. A limited number of observations indicated that the appearance of antigenic variants in cultures developing from single cells is preceded by reorganization in the nuclear apparatus of all or nearly all of the individuals in the population.

ONE HUNDRED AND SEVENTY-EIGHTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY  
BUILDING, PHILADELPHIA, PA., FEBRUARY 27, 1945.

**FULMINATING OBSTRUCTIVE LARYNGITIS AND SEPTICEMIA DUE TO HEMOPHILUS INFLUENZAE, TYPE B.** *John Eiman and Russel Fowler*, Department of Pathology, Abington Memorial Hospital, Abington, Pa.

During the past few years a severe and often fatal form of laryngitis with bacteremia caused by *Hemophilus influenzae* type B has been reported among young children from 18 months to 11 years of age.

At Abington Memorial Hospital we have had three such cases, two in 1938 and one in December of 1944.

The clinical pictures in all three cases were essentially the same and were as follows: Difficulty in swallowing, sore throat, and temperature from 104 to 105 F appeared during an apparently upper respiratory infection. Dyspnea started abruptly and increased within a few hours to such a degree that tracheotomy had to be done. The children were prostrated and showed evidence of an overwhelming infection. The epiglottis was markedly edematous and bright red in color, and the opening to the larynx was narrowed to a small irregular slit. The inflammation of the epiglottis was responsible for the partial obstruction and dyspnea.

Cultures taken from the mucus in the pharynx or in the trachea and the blood in all three cases showed *Hemophilus influenzae* type B.

The results in these three cases were as follows:

No. 1. Died—Dead on arrival at the hospital. Tracheotomy not performed. Sulfonamide drug and serum not administered.

No. 2. Died—Moribund on admission. Tracheotomy performed. Sulfonamide drug and serum not administered.

No. 3. Recovered—Tracheotomy performed. Sulfanilamide and anti-*H. influenzae* serum administered.

**AN IMPROVED LIQUID CULTURE MEDIUM FOR THE GROWTH OF HEMOPHILUS PERTUSSIS.** *W. F. Vervey and Dorothy Sage*, Department of Bacteriology, Medical

Research Division, Sharp and Dohme, Glenolden, Pa.

A casein hydrolyzate liquid culture medium for the growth of *Hemophilus pertussis* has been described by Hornibrook. In attempts to prepare pertussis vaccine in liquid media, Hornibrook's formula was tested, and, although found capable of supporting the growth of freshly isolated strains, was not satisfactory for vaccine production. The medium was then modified by the removal of sodium chloride, calcium chloride, and sodium carbonate, and by increasing the concentrations of casein hydrolyzate, potassium phosphate, and nicotinic acid. The modified medium was capable of supporting growth to about twice the turbidity of the original Hornibrook medium. In confirmation of Hornibrook's work it was found that when an extract of human blood cells was added to either the original or modified medium the rate of growth and the ultimate density of the cultures were increased. Organisms that were grown in the modified medium containing blood cell extract were proved to maintain their phase I characteristics and virulence for mice. Whole culture vaccine prepared from *H. pertussis* grown in this medium was found to have antigenicity at least equivalent to that of vaccine prepared from organisms washed from Bordet-Gengou medium. This vaccine, when injected into humans, produced local and systemic reactions no greater than those caused by vaccine prepared from Bordet-Gengou cultures.

**THE USE OF HEMOPHILUS PERTUSSIS AGGLUTINOGEN IN THE PREPARATION OF HYPERIMMUNE HUMAN SERUM FOR THE TREATMENT OF WHOOPING COUGH.** *H. Felton, J. Smolens, S. Mudd, M. Carr, I. Lincoln, and L. Walker*, Departments of Bacteriology and Pediatrics of the University of Pennsylvania and Children's Hospital of Philadelphia, Pa.

The immunizing possibilities of purified phase I *Hemophilus pertussis* agglutinin have been suggested by the results of preliminary laboratory investigations and later

clinical use in humans. This agglutinin produces clear-cut skin reactions when used as a test for susceptibility to whooping cough, and also produces a marked rise in agglutinin titer of individuals showing an immune reaction to the test dose. It is possible to reverse the skin reaction and to produce a detectable agglutinin titer in a susceptible individual by repeated intradermal doses of agglutinin.

A group of twelve new donors of the Philadelphia Serum Exchange were immunized fully by the intradermal use of pertussis agglutinin. Eight donors were available for use in the serum pool after receiving a series of three doses of 10 units of acid-extracted agglutinin at weekly intervals.

The so-called hyperimmune titer was maintained in these donors by 1-unit doses of agglutinin given at four- to five-week intervals.

A pool of serum was prepared from the blood of the donors who had been immunized with agglutinin. The final titer of this pool was 1:2,560. This is the usual level found in pools of serum from donors who have received the routine Sauer vaccine immunization.

This serum was used with satisfactory results in the treatment of fifteen children with clinical whooping cough. The results were excellent and were comparable to those obtained by the use of the usual hyperimmune serum.

### SOUTHERN CALIFORNIA BRANCH

UNIVERSITY OF SOUTHERN CALIFORNIA, LOS ANGELES, CALIFORNIA,  
FEBRUARY 27, 1945

#### OBSERVATIONS ON THE OCCURRENCE AND METABOLISM OF LIPOCLASTIC ANAEROBES.

*William D. Rosenfeld*, Scripps Institution of Oceanography, University of California, La Jolla.

The neutral red base method proposed by Knaysi has permitted the detection of anaerobic lipolysis. Fat-splitting bacteria have been demonstrated in tar sand, asphalt, paraffin earth, crude oil, marine mud, and oil well brine samples; these organisms have actively hydrolyzed a variety of lipid materials including oils, waxes, glycerides, and simple esters. Fatty acids have been liberated from a series of triglycerides ranging from triacetin through tristearin. The anaerobic character of such cleavage processes is borne out by the fact that lipolyses occurred at Eh levels more negative than -100 millivolts.

Hydrolytic products are utilized by both lipoclastic and nonlipoclastic microorganisms. Evidence indicates that such products are important in the respiratory activities of the bacteria.

The microbial utilization of lipids is not precluded by a lack of lipolytic powers. Several organisms have been shown to oxidize intact glycerides and esters under anaerobic conditions.

#### INCREASED GERMICIDAL EFFICIENCY OF IODINE IN THE PRESENCE OF AN OXIDATION-REDUCTION SYSTEM. *A. J. Salle*, Department of Bacteriology, University of California, Los Angeles.

In the absence of organic matter, iodine killed *Staphylococcus aureus* in a dilution of 1:20,000 and *Eberthella typhosa* in a dilution of 1:17,500 in 10 minutes at 37 C.

Iodine dissolved in a 1:3,000 dilution of an oxidation-reduction solution (composed of a mixture of one mole each of manganous sulfate and ferric sulfate) killed *S. aureus* in a dilution of 1:80,000 and *E. typhosa* in a dilution of 1:60,000, in the absence of organic matter.

A dilution of 1:2,000 of the O-R solution killed embryonic chick heart tissue fragments in 10 minutes at 37 C. Iodine dissolved in a 1:3,000 O-R solution killed the tissue fragments in a dilution of 1:3,750 of iodine.

Toxicity indexes may be calculated by dividing the highest dilution of iodine required to kill the tissue by the highest dilution required to kill the test organism. The toxicity index of iodine + 1:3,000 O-R solution was 0.05 for *S. aureus* and 0.06 for *E. typhosa*. The toxicity index of iodine alone was 0.2 for *S. aureus* and 0.23 for *E.*



*typhosa*. The smaller the index the more nearly perfect the germicide.

When iodine was dissolved in a 1:3,000 O-R solution composed of manganous and ferric chlorides instead of the sulfates, the toxicity indexes were 0.042 for *S. aureus* and 0.1 for *E. typhosa*.

#### THE OCCURRENCE OF HYDROGEN-PRODUCING BACTERIA IN MARINE MATERIALS.

*Josephine Beckwith Senn*, Scripps Institution of Oceanography, University of California, La Jolla.

The production of hydrogen by microbial activity in a marine environment may be of considerable importance in the hydrogenation of organic matter by converting the latter into substances which are more hydrocarbonlike in character. Hydrogen-producing microorganisms have been demonstrated in various marine muds, oil well cores, natural rock asphalt formations, and other marine materials both recent and ancient. According to preliminary estimates, these organisms appear to be relatively abundant. The minimum dilution method has shown from 1,000 to 10,000 hydrogen-producing organisms per gram (dry basis) of the estuarine muds which have been studied. Microorganisms which produce hydrogen seem to be geographically widespread in distribution. They have been detected in many marine deposits collected along the California coast, in brine from California petroleum fields, in oil well samples from Oklahoma, and in bituminous deposits from Kentucky. Microbiological activity has yielded hydrogen from cellulose, peptone, oleic acid, sperm oil, coconut oil, and glycerol substrates. The gas produced from these materials has been found to contain from less than 1 per cent to as much as 67 per cent hydrogen, with glycerol as the substrate giving the highest hydrogen yields to date.

#### THE IMPORTANCE OF AIR-DECONTAMINATION IN A COMMUNICABLE DISEASE HOSPITAL.

*C. F. Pait*, Los Angeles County Hospital.

The diseases which may be spread by the aerial route were listed and discussed. The various existing methods for isolating communicable disease patients were outlined, and the inadequacies of some of these meth-

ods with respect to air-borne contagion were pointed out. A brief review of methods available for control of air-borne diseases was presented.

#### EFFECT OF BACTERIAL ACTIVITY ON PETROLEUM HYDROCARBONS.

*Claude E. ZoBell*, Scripps Institution of Oceanography, University of California, La Jolla.

Nearly all hydrocarbons which have been tested have proved to be susceptible to bacterial oxidation under aerobic conditions. A few anaerobes, notably certain sulfate reducers, are able to oxidize long-chain, aliphatic hydrocarbons. The bacteria utilize the hydrocarbons as a sole source of carbon or energy in appropriate mineral salts solutions. Emulsifying the hydrocarbons or adsorbing them on solid surfaces promotes the growth of bacteria.

The bacterial oxidation of hydrocarbons has been observed at temperatures ranging from 0 to 60 C and at salinities ranging from near zero to as high as 300,000 ppm. The process is generally inhibited by reducing conditions, the presence of hydrogen sulfide, or by relatively low concentrations of vanadium, zinc, or molybdenum.

By virtue of their preference for long-chain and branched-chain hydrocarbons, microorganisms tend to decrease the density of crude oils under certain conditions. The greatest observed decrease has been on a sample of California crude oil, the density of which was reduced from 0.849 to 0.802. Bacteria also have a tendency to attack preferentially compounds in crude oil containing nitrogen, sulphur, or oxygen.

The formation of hydrocarbons ranging from C<sub>20</sub> to C<sub>35</sub> by species of *Desulfovibrio* growing on fatty acids has been observed.

#### SHIGELLA AND SALMONELLA ENCOUNTERED IN SOUTHERN CALIFORNIA 1943-1944.

*John F. Kessel*, *Margaret Parrish*, and *Korine Cavell*, School of Medicine, University of Southern California, and Los Angeles County Hospital.

With the use of serums prepared in this laboratory from cultures of *Shigella* furnished by the Army Medical School, the following strains have been encountered in the diagnostic routine of the Los Angeles County Hospital.

*Shigella paradysenteriae*

Flexner	Boyd
I—57	I— 7
II— 1	II— 3
III— 9	III— 3
IV—30	D19—12
V— 9	143— 4
VI—14	274—12
Sonne—83	madampensis—12

It is of interest to note, even though Boyd reports some of these strains as being rare, that all have been encountered in Southern California. In the future we expect to compare results with the recent classification proposed by Weil *et al.* (1944).

During the same period, *Salmonella* strains encountered in the Los Angeles County Hospital, together with a few additional strains provided by other local laboratories, have been typed by the Kaufmann-White method using sera supplied by the Army Medical School. The following types have been encountered:

*Salmonella* types

<i>typhi-murium</i> . . . . .	42	<i>bareilly</i> . . . . .	2
<i>newport</i> . . . . .	18	<i>oregon</i> . . . . .	1
<i>derby</i> . . . . .	7	<i>meleagridis</i> . . . . .	1
<i>montevideo</i> . . . . .	4	<i>london</i> . . . . .	1
<i>panama</i> . . . . .	3	<i>litchfield</i> . . . . .	1
<i>saint-paul</i> . . . . .	2	<i>paratyphi A</i> . . . . .	1
<i>javana</i> . . . . .	2	<i>uganda</i> . . . . .	1
<i>paratyphi B</i> . . . . .	2	<i>cholerae-suis</i> . . . . .	1
<i>oranienburg</i> . . . . .	2	<i>eastbourne</i> . . . . .	1
<i>anatum</i> . . . . .	2	<i>newington</i> . . . . .	1

It is of interest to note that eleven of these types have recently been reported by Hinshaw (1944) as present in avian salmonellosis in California.

**PROTEUS SPECIATION.** Milton G. Levine and Robert E. Hoyt, Institute of Experimental Medicine, College of Medical Evangelists.

Despite recent suggestions in the literature that new species of *Proteus* be created because of the inadequacy of the present classification, we find that Hauser's original species, *Proteus vulgaris* and *Proteus mirabilis*, are adequate and the characteristics described constant. On the basis of the following reactions, we have found *Proteus mirabilis* to be by far the most common *Proteus* species occurring in nature:

Characteristics of *Proteus*

	MAL-TOSE	SUCROSE	IN-DOLE	ALPHA-METHYL-GLUCOSIDE	SUCROSE AGAR SLANT
<i>Proteus vulgaris</i>	AG	AG	+	AG	Acid
<i>Proteus mirabilis</i>	—	Slow AG	—	—	—
<i>Proteus ammoniae</i>	—	Slow AG	—	—	—

AG = acid and gas formed. All produced acid and gas from glucose.

*Proteus ammoniae* cultures obtained from Dr. Magath proved to be identical with *Proteus mirabilis* as shown above.

## KENTUCKY BRANCH

LEXINGTON, KENTUCKY,

OCTOBER 28, 1944<sup>1</sup>

THE IDENTITY OF UDDER STREPTOCOCCI IN SEVEN KENTUCKY DAIRY HERDS. H. B. Morrison and F. E. Hull, Dairy Section and Animal Pathology Department, University of Kentucky.

Milk samples were obtained at intervals during 1943 and 1944 from seven Kentucky dairy herds. Of 335 cows in these herds, 176 (52.6%) were giving off streptococci in their milk. One hundred forty-four cultures from 139 cows were identified by means of biochemical characteristics and the Lance-

field technique. Of these, 102 (70.8%) proved to be *Streptococcus agalactiae*, 41 (28.5%) *Streptococcus uberis*, and 1 (0.7%) *Streptococcus dysgalactiae*.

These percentages agree in general with reports published by several other investigators. However, *S. agalactiae* was the major infectious agent in only three of the seven herds. In each of these three herds, which included the two largest herds in the study, more than 90 per cent of the cultures identified were *S. agalactiae*. *S. uberis*

infection predominated in the other four herds. From one of these herds no cultures of *S. agalactiae* were identified.

It appears more than mere coincidence that the three herds in this study having a high percentage of infections from *S. agalactiae* had only average or poorer quality of labor for milking and other herd care, whereas the herds with a low percentage of *S. agalactiae* infection as compared with *S. uberis* had much better than average quality of labor.

**PROTEIN FORTIFICATION OF CORN DISTILLERS' DRIED SOLUBLES AND DARK DRIED GRAINS.** J. K. Woods, S. L. Adams, and W. H. Stark, Joseph E. Seagram & Sons, Inc.

Stillage, the residue from the distillation of fermented mash, is screened. The screenings are dried to produce light distillers' dried grains. The thin stillage is concentrated in multiple effect evaporators to a syrup which is dried on drum driers to produce distillers' dried solubles. Dark dried grains are produced by combining the syrup and press cake prior to drying. Both light and dark dried grains are marketed as cattle feeds. Their main value lies in their protein content. Solubles are used as a vitamin supplement in poultry rations, but their protein content is also important.

The method employed to increase the protein consisted of the addition of an inexpensive nitrogen compound to the fermenter mash and its utilization to produce yeast amino acids and proteins. The nitrogen source had an adverse effect upon alcohol yields. Urea proved to be the best nitrogen source although the addition of ca 0.9 g per L decreased the alcohol yield ca 0.1 p.g. per bu. More than 90 per cent of the urea added was converted into protein. Practically all of the synthesized protein was recovered in the dried solubles. Under normal circumstances the increased value of the higher protein product more than offsets the loss in yield and the cost of the urea.

These data are based on laboratory fermentations; no plant trials have been made.

**A SEROLOGICAL STUDY OF MEMBERS OF THE PSEUDOMONAS GENUS.** J. Munoz, M. Scherago, and R. H. Weaver, Department

of Bacteriology, University of Kentucky, Lexington, Kentucky.

A study has been made on a representative group of strains of *Pseudomonas* to determine whether there is any serological relationship among strains of this genus. The most significant findings follow.

A group of monotrichic strains that had the morphological and biochemical characteristics of *P. aeruginosa* could be differentiated into two distinct serological groups, one of which was homogeneous and the other heterogeneous. A lophotrichic strain that had the same biochemical characteristics showed a strong flagellar relationship to the heterogeneous group. These findings are in agreement with those of Acki (1926), Kan-zaki (1934), Harris (1940), and Elrod and Braun (1942), who reported serological differences among strains of *P. aeruginosa*.

*P. caviac*, *P. fragi*, *P. graveolens*, *P. mucidolens*, and *P. pavonacea* species, which are readily differentiated by their biochemical characteristics, could also be differentiated serologically.

*P. fluorescens*, *P. mildenbergii*, *P. ovalis*, and *P. putida*, species which can not be satisfactorily differentiated by means of their biochemical characteristics, could be very easily differentiated by serological means.

It may be concluded that serological methods are essential for the identification and classification of members of the *Pseudomonas* genus.

**PREPARATION OF SPECIFIC SALMONELLA SERUMS.** P. R. Edwards, University of Kentucky.

**ETHANOL FERMENTATION OF DEHYDRATED SWEET POTATOES—NUTRIENT STUDY.** L. C. Hao and M. E. Delahanty, Joseph E. Seagram & Sons, Inc.

The use of nutrients for improving the yield obtained from the alcoholic fermentation of dehydrated sweet potatoes was studied. The addition of mineral salts such as ammonium sulfate, calcium phosphate, and potassium monohydrogen phosphate to the water mash of dehydrated sweet potatoes did not increase the alcohol yield. Actually, for unknown reasons, the yield was reduced.

Mylase, a mold-bran preparation, was proved to be both an efficient saccharifying agent and a good nutrient. When 3 to 4 per cent of mylase, based on the weight of dehydrated sweet potatoes, was used for saccharification, the yield of alcohol obtained was 6.4 p.g. per bu on a dry basis. The sweet potato has a starch content of 73.0 per cent, dry basis. The yield with mylase was approximately 10 per cent higher than the yield of 5.8 p.g. per bu obtained

from corresponding water mash control fermentations, and a trifle higher than the yield of 6.31 p.g. per bu obtained from stillage mash control fermentations.

Sterilized raw wheat bran and sterilized mylase were partially effective in improving the yield. These data indicate the presence of both heat-stable and heat-labile growth factors or enzymes other than amylase which are responsible for the improvement of alcohol yield.

### NEW YORK CITY BRANCH

TEACHERS COLLEGE, COLUMBIA UNIVERSITY,

MARCH 13, 1945

DIAGNOSTIC PROBLEMS OF ENTERIC ORGANISMS (*ESCHERICHIA*, *SHIGELLA*, *SALMONELLA*) DUE TO THEIR BIOCHEMICAL AND SEROLOGICAL RELATIONSHIPS. *Ivan Saphra*, Beth Israel Hospital, New York City.

Enterobacteriaceae (*Salmonella*, *Shigella*, *Escherichia*, *Proteus*, etc.) form an integrating series of types, related in their cultural, biochemical, antigenic, and pathogenic properties. Transitional forms and serological interrelationships between types, groups, species, and genera are frequent. Of particular interest are the serological cross reactions which center around the O-antigens of *Salmonella enteritidis* (I-VI-XIV-XXV) and of related types. The ensuing genetic, taxonomic, and diagnostic problems were demonstrated, and methods of solving them were indicated.

THE ACTIVITY OF PENICILLIN IN THE EXPERIMENTAL PNEUMOCOCCUS PNEUMONIA OF MICE. *R. J. Schnitzer*, *N. Ercoli*, *M. Buck*, and *D. R. Kelly*, Hoffmann-LaRoche, Inc., Nutley, N. J.

Mice were infected intranasally with type I and type II pneumococci, using the Shope method according to Neufeld and Etlinger Tulczynska. This infection leads to pneumonia of both lungs. It usually does not respond to chemotherapeutic treatment, e.g., treatment with sulfonamides, but reacts readily to the administration of penicillin. The minimal active dose of sodium penicillin injected 2 to 4 times subcutaneously was a total of 800 to 1,000 units

per 20 g in type I infections; in infections with type II pneumococci a total of 1,800 to 2,000 units per 20 g was required. Oral administration of penicillin was as active as the treatment by the parenteral route.

THE FATE OF *TREPONEMA PALLIDUM* IN RABBITS TREATED WITH PENICILLIN. *N. Ercoli* and *E. M. Harker*, Hoffmann-LaRoche, Inc., Nutley, N. J.

Although the clinical problem of permanent cure of syphilis in man will require observations of a great number of patients over a very long period of time, it is possible to decide the very similar question of the sterilizing effect of a drug, e.g., penicillin, comparatively quickly in experimental syphilis of rabbits.

From our recent publications (Proc. Soc. Exptl. Biol. Med., **57**, 4, 1944) it is known that a single dose of 33,000 units per kg given intravenously causes disappearance of the spirochetes in less than 18 hours. In an addendum we noted that even much higher doses of penicillin were not sterilizing; the details of these experiments are presented.

The results of the lymph node transfer, as well as the reinfection test of the apparently healed rabbits, revealed that virulent spirochetes survived in the organs of rabbits, even though the doses of penicillin used were much higher than is required to give prompt clinical healing.

Twelve to eighteen times the dose sufficient to induce disappearance of spirochetes and dissolution of the lesion was not sterilizing.

These experimental data suggest that only doses much higher than those required to obtain clinical healing might produce sterilizing cure in spirochetal infections.

A SUGGESTED METHOD FOR THOROUGH TESTING OF ANTISEPTIC FABRICS. *Louis C. Barail*, United States Testing Co., Inc., New York City.

## MICHIGAN BRANCH

DETROIT, MICHIGAN,

MARCH 16, 1945

RAPID IDENTIFICATION OF SHIGELLA BY SEROLOGIC METHODS. *W. Ferguson and M. Carlson*, Bureau of Laboratories, Michigan Department of Health, Lansing.

The contributions of Boyd (1932, 1936), Wheeler (1944), and Weil, Black, and Farsetta (1944) to our knowledge of the paradysentery group of *Shigella* were reviewed and the differences in the antigenic schema proposed by Wheeler and by Weil and colleagues were discussed.

TABLE 1  
Number of strains

	MICHIGAN	NEW MEXICO
Type I (V) .....	66	0
Type II (W).....	28	8
Type III (Z)....	8	2
Type IV (Boyd 103)	11	2
Type V (Boyd P-119)	2	2
Type VI (Boyd 88-Newcastle).....	3	5
"X" .....	0	0
"Y".....	2	2
Boyd P-288. ....	0	1
Totals... ..	120	22

Following the methods of Wheeler, the authors prepared typing sera for Flexner and Boyd types of *Shigella paradysenteriae* and applied them to type determination of Michigan and New Mexico strains. The latter were furnished by courtesy of the New Mexico State Department of Health. Table 1 shows the type determinations made.

Absorbed sera for identification of *Shigella ambigua*, *Shigella sonnei*, and *Shigella alkalescens* have been prepared and used by the authors, together with polyvalent

serum covering the paradysentery types I to VI and including "Y" and "X." No disagreement has been found between slide agglutination identification and biochemical and tube agglutination methods.

THE USE-DILUTION METHOD OF TESTING DISINFECTANTS. *W. L. Mallmann and Marjory Hanes*, Department of Bacteriology and Public Health, Michigan State College, East Lansing, Michigan.

The F. D. A. procedure for testing disinfectants is not satisfactory because it is not measuring the actual dilution recommended for use. The following procedure is suggested as a means of checking disinfectants in use dilutions although greater dilutions may be tested.

Sterile glass rods,  $\frac{1}{4}$ " x 1" with a small eye at one end for handling, are dipped in a 24-hour culture of the test organism. The seeded rods are dried on sterile filter paper in a petri dish for 30 minutes. The seeded rods are placed in the test solution at 20 C for 1, 5, 10, and 30 minutes. Upon removal, each rod is rinsed in a tube of sterile water or neutralizing solution and then transferred to a tube of standard broth. After the broth has been shaken, 1-ml portions are plated. The tubes containing the rods are incubated 24 hours and replated if the tubes are not turbid from growth.

THE USE OF PERRY'S MEDIUM FOR THE ISOLATION OF FECAL STREPTOCOCCI—PRELIMINARY REPORT. *Elizabeth J. Cope*, Detroit Department of Health.

Five hundred fourteen consecutive fecal specimens submitted to the Enteric Division of the Detroit Department of Health Laboratory were cultured in Perry's medium. Streptococci were recovered from 80.2 per cent. Of the streptococci isolated 92.1 per cent were not killed when heated at 60 C in a water bath for one hour.

# CAN CHEMOTHERAPY BE EXTENDED TO INCLUDE THE INTRACELLULAR DISEASE AGENTS?<sup>1</sup>

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The present limits of successful chemotherapy do not quite coincide with the line of division between extracellular and intracellular disease agents. Thus chemotherapeutic benefit in experimental murine typhus has recently been reported by Moragues, Pinkerton, and Greiff (1944), and in human epidemic typhus by Yeomans and others (1944). Chemotherapeutic benefit is well authenticated for infections with viruses of lymphogranuloma venereum, mouse pneumonitis, trachoma, inclusion conjunctivitis, and most recently is reported for experimental ornithosis and psittacosis (Heilman and Herrell, 1944). Yet the pathogenic rickettsiae and the viruses of the lymphogranuloma-psittacosis group are obligate intracellular parasites. Nor is the limit of current chemotherapeutic effectiveness coincident with the limit of simple cellular organization of the disease agent (Mudd and Anderson, 1944); vaccinia virus and even coliphage have been shown by electron microscopy to possess cell-wall, inner protoplasm, and even finer structural differentiation; yet no authenticated success in chemotherapy with viruses other than those cited above is known. It is interesting and possibly significant that those intracellular agents which now respond to chemotherapy are the rickettsiae, which are intermediate in size between pathogenic bacteria and the viruses, and members of the lymphogranuloma-psittacosis group, which are the very largest of known viruses.

Several approaches to a chemotherapy of intracellular disease agents can be formulated:

(1) *Direct chemical action on the disease agent.* The long list of empirical trials has recently been reviewed by Kramer, Geer, and Szobel (1944). See also Parker and Diefendorf (1944) and Klein, Kalter, and Mudd (1945).

(2) *Interference with the key-to-lock relationship of virus to susceptible host cell.* This is precisely what specific antiphage (antiviral) antibody does when used prophylactically, as the experiments of Kalmanson and Bronfenbrenner (1943) on reversible serum inactivation of bacterial virus show. It is hard to imagine, however, what more (if as much) could be expected in this connection from any chemical agent than is now achievable by specific antiserum—namely, prophylaxis which is efficient, but therapeutic action which is very limited indeed.

(3) *Rational investigation of cell metabolic systems and their selective inhibition.* This approach seems to the writer incomparably the most promising one. The essential attribute of a chemotherapeutic agent is a selective action on a component essential to the continued existence of the parasite within its host. Sir Henry Dale in a recent review of the history of chemotherapy (1943) clearly

<sup>1</sup> Presidential Address of the Society of American Bacteriologists given with additions before the New York City Branch, December 28, 1944, the Maryland Branch, April 5, the Eastern Pennsylvania Branch, May 22, and the Central New York Branch, May 26, 1945.

points out that in a large proportion of chemotherapeutic cures "the infection is brought to an end by stopping the further multiplication of the parasites, rather than by killing them outright. Another factor in an effective chemotherapeutic action . . . [is] the need for a sufficiently prolonged and continuous action. This need is almost implied in the conception of the process as essentially an arrest of the multiplication of the parasites rather than an immediately lethal action on them. What is required is not the sudden attainment of a concentration sufficient to kill most of the parasites, at the risk of a concomitant injury to the host's tissues, but the long-continued maintenance of a much lower and safer concentration, just sufficient to suppress the propagation of the parasites, without harming the cells of the host."

Evidence with respect to the mode of action of successful chemotherapeutic agents is in agreement with the original conception of Ehrlich and with Dale's conclusion that they act essentially by arrest of the multiplication of the parasites. A definition of objective then becomes possible. For successful chemotherapy of an infection due to an intracellular parasite there may be sought a *metabolic inhibitor or inhibitors which selectively inhibit a reaction or reactions essential to the intracellular multiplication of the parasite*, but (at least temporarily) inessential to the survival of the host cell.<sup>2</sup>

Concerning the mechanism of action of the sulfonamides two theories are current:

(1) The Woods-Fildes theory that sulfonamides inhibit an enzyme or enzymes concerned with the anabolism of *p*-aminobenzoic acid as an essential metabolite.

(2) The theory proposed chiefly by Sevag and coworkers (1942) that sulfonamides act by inhibiting in the susceptible bacteria certain respiratory enzymes which normally mediate reactions yielding the energy essential to bacterial cell division.

The fundamental proposition that substances similar to but not identical in configuration with particular components in a metabolic reaction can compete for essential reaction sites and thereby inhibit the metabolic reaction is not questioned. (See Fildes, 1940, 1941; Woods, 1940; Roblin *et al.*, 1945, for references.) This fundamental principle is valid and applicable, however, whether the enzyme inhibitor resembles and competes with a substrate, or, in the case of the oxidative enzymes, with a coenzyme. The Woods-Fildes theory has been of the utmost importance in emphasizing configurational correspondence as a basis for enzyme inhibition in chemotherapy, and in stimulating the discovery of new instances and applications (e.g., see McIlwain, 1944; Woolley, 1944). In the writer's belief, however, the balance of evidence very definitely now indicates that in the special case of the mechanism of sulfonamide action

<sup>2</sup> An increased resistance to experimental poliomyelitis in animals on thiamine-deficient diets seems to be well authenticated (Foster, Jones, Henle, and Dorfman, 1942, 1944a, 1944b; Rasmussen, Waisman, Elvehjem, and Clark, 1944; Editorial, *J. Am. Med. Assoc.*, 1945). Could it be that mechanisms essential to the intracellular multiplication of this parasite are peculiarly sensitive to thiamine deficiency? Could mechanisms essential to parasite reproduction in host cells eventually prove to be peculiarly vulnerable to specific deficiencies, whether produced by dietary restrictions or by administration of drugs (Woolley, 1944)?

the critical competition is with respiratory coenzymes for sites on the oxidative enzyme proteins rather than for a hypothetical enzyme mediating *p*-aminobenzoic acid anabolism. The subject is comprehensively reviewed by Sevag and coworkers (1942, 1944, 1945) and Henry (1943, 1944).

Examination of such literature as is available on the mode of action of chemotherapeutic agents leads, at least tentatively, to the challenging conclusion that in cases in which critical enzyme inhibitions correlated with inhibition of growth have been identified experimentally, these critical sites of chemotherapeutic action have been found to be *within the system of respiratory enzymes which mediate the aerobic and anaerobic oxidation of glucose and its derivatives*.

The respiratory enzymes are proteins reversibly combined with coenzymes; the coenzymes contain as essential structural units certain components of the B vitamin complex, notably nicotinic acid amide, riboflavin, and thiamine; or, in the case of the cytochrome-cytochrome oxidase system, the iron porphyrin compound *heme* is the prosthetic group.

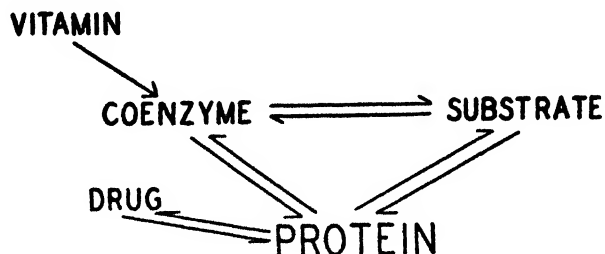


FIG. 1. SCHEMA SHOWING INTERRELATIONSHIPS BETWEEN GROWTH ACCESSORY, SULFONAMIDE DRUG, RESPIRATORY ENZYME, AND SUBSTRATE

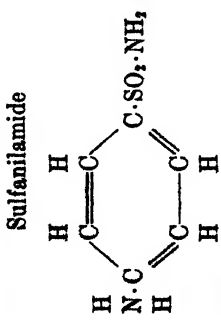
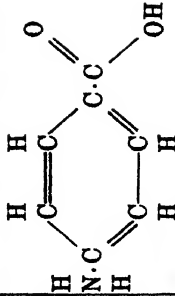
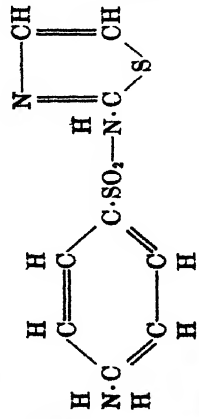
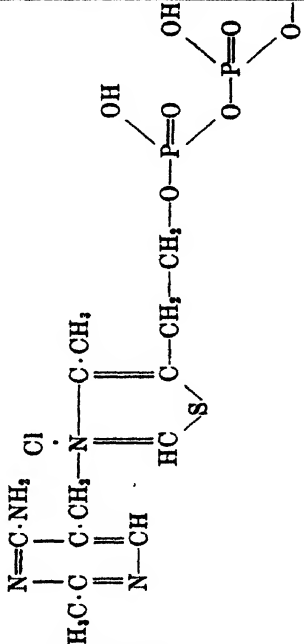
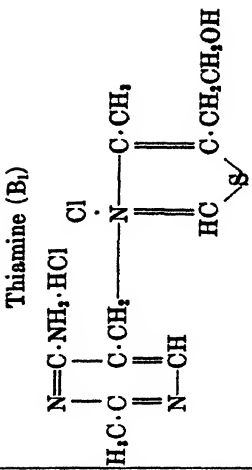
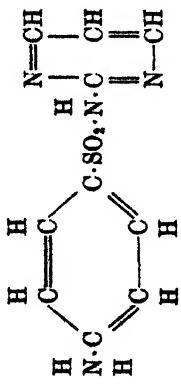
These relationships may be represented schematically as in figure 1. Thiamine for instance becomes by phosphorylation cocarboxylase (thiamine diphosphate) and riboflavin becomes riboflavin phosphoric acid. The enzyme carrier protein combines reversibly with its coenzyme and specific substrate, thus enabling the substrate to reduce the coenzyme, the substrate itself being oxidized.

We suppose that sulfanilamide has affinity for the respiratory enzyme protein<sup>3</sup>

<sup>3</sup> In this connection the similarity of structure of sulfanilamide to other pharmacologically active substances is of interest. Compare for instance the structure of *p*-aminobenzenesulfonamide with that of the *p*-aminophenol derivatives (antipyretics and analgesics), procaine, ethyl and butyl *p*-aminobenzoates (local anesthetics), carbarsone (amebacide), and trypanamide (trypanocide). It is suggested that widely distributed reaction sites exist with which compounds possessing the general configuration of *p*-aminobenzenesulfonamides, *p*-aminobenzoates, and *p*-aminophenylarsonates and their appropriate derivatives can combine reversibly with a resulting depressant effect. It is suggested that these reaction sites on oxidative enzyme proteins are of critical significance in chemotherapy.

However, in calling attention in this address to interrelationships based upon configurational correspondence, no implication is intended that all cases of inhibition and antagonism involve such configurational correspondence, which is obviously not true. In enzyme chemistry as in immunochemistry, however, the interrelationships depending upon specific configuration (Sevag, 1945) are perhaps more interesting and more stimulating to further discovery than those involving only nonspecific relationships.



CHEMOTHERAPEUTIC	COENZYME	GROWTH ACCESSORY SUBSTANCE
<p>Sulfanilamide</p> 		<p><i>p</i>-Aminobenzoic acid (?) *</p> 
<p>Sulfathiazole</p> 	<p>Cocarcboxylase</p> 	<p>Thiamine (B1)</p> 
<p>Sulfadiazine</p> 		

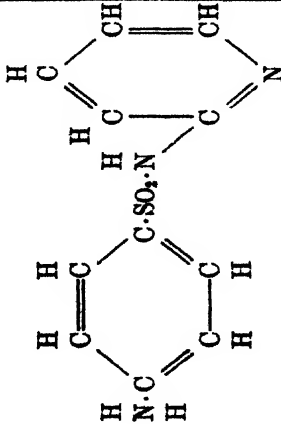
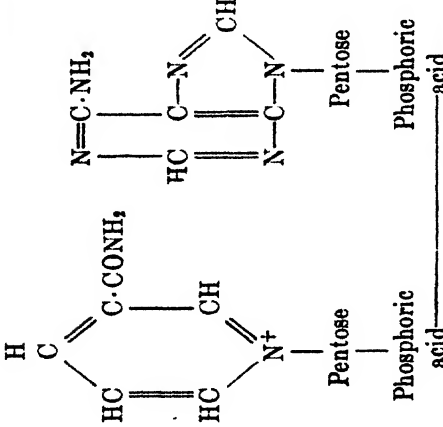
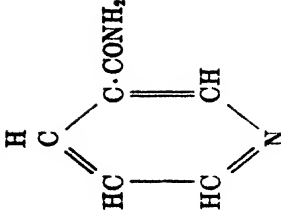
Sulfapyridine	Cozymase	Nicotinic acid amide
		

FIG. 2. CHEMICAL STRUCTURE OF GROWTH-ACCESSORY SUBSTANCES KNOWN TO BE PRECURSORS OF RESPIRATORY COENZYMES AND SULFONAMIDE DRUGS HAVING CONFIGURATIONS IN COMMON WITH THE COENZYMES.

\*The writer does not believe *para*-aminobenzoic acid to be a growth accessory for pathogenic bacteria.

and is supplemented in the newer sulfonamides by the N<sup>1</sup> substituted group (pyridine, thiazole, pyrimidine) which structurally resembles the coenzyme or part of it, and competes with the coenzyme for its reaction site on the enzyme protein. (See figures 1, 2, and 3.) The proposal herewith suggested is that the sulfanilamide and the N<sup>1</sup> substituted group are capable of combining, respectively, at two reaction sites, in appropriate steric relationship, on the enzyme protein. Such a proposal is not without analogy since dipeptidase action is believed to depend on three reaction sites on the enzyme in very definite steric relationship (Schmidt, 1938). The drug-protein or drug-protein-coenzyme complex we suppose to be inactive enzymatically. Quantitative antagonism between drug and coenzyme thus becomes understandable, and similarly antagonism between vitamin and drug, since the vitamin is a precursor of the corresponding coenzyme. The superiority of sulfapyridine, sulfathiazole, and sulfadiazine over sulfanilamide is intelligible in these terms but difficult to account for on the *p*-aminobenzoic acid theory.

The following critical sites of chemotherapeutic action may be cited as indicated by experiment.

Dorfman and Koser (1942) showed that the respiration of dysentery bacilli was increased by nicotinamide and inhibited by sulfapyridine (cf. figure 2). MacLeod (1939) found that the dehydrogenase activity of pneumococci for glycerol, lactate, and pyruvate was inhibited by sulfapyridine. Acquisition of sulfapyridine fastness was associated with marked loss of dehydrogenase activity for these compounds. Fox (1942) working with isolated enzyme systems of *Escherichia coli* demonstrated inhibition of lactate, aerobically, and dismutation of pyruvate, anaerobically.

*Flavoprotein dehydrogenases* (riboflavin phosphoric acid proteins). Sevag and Green (1944) found that growth of certain strains of *Staphylococcus aureus* in the absence of added glucose is inhibited by sulfathiazole, and that this inhibition is antagonized by riboflavin. Atabrine has been shown by Haas (1944), working with isolated enzyme systems of yeast, to inhibit oxidation mediated by flavoprotein, and this inhibition is antagonized by the coenzyme (cf. figure 3). Selective inhibition of flavoprotein enzyme systems by atabrine has been indicated by tissue respiration experiments (Wright and Sabine, 1944).

*Carboxylase* (thiamine pyrophosphate protein). Sevag, Shelburne, and Mudd (1942, 1945) found that sulfathiazole selectively inhibits the action of carboxylase from both yeast and staphylococcus; this inhibition is antagonized by cocarboxylase (cf. figure 2). Penicillin has been shown by Welshimer, Krampitz, and Werkman (1944) to inhibit selectively the dismutation of pyruvic acid.

*Cytochrome-cytochrome oxidase* system (heme-containing enzymes). Cytochrome oxidase has been shown by Haas (1944) to be inhibited by atabrine. Zephiran has been shown by Sevag and Ross (1944) to inhibit the cytochrome-cytochrome oxidase system of yeast cells, and this action is correlated with inhibition of growth. These are probably instances of nonspecific inhibition.

The Pasteur effect has been shown to be strongly inhibited by certain substituted guanidines and amidines which are powerful trypanocides (Dickens, 1939).

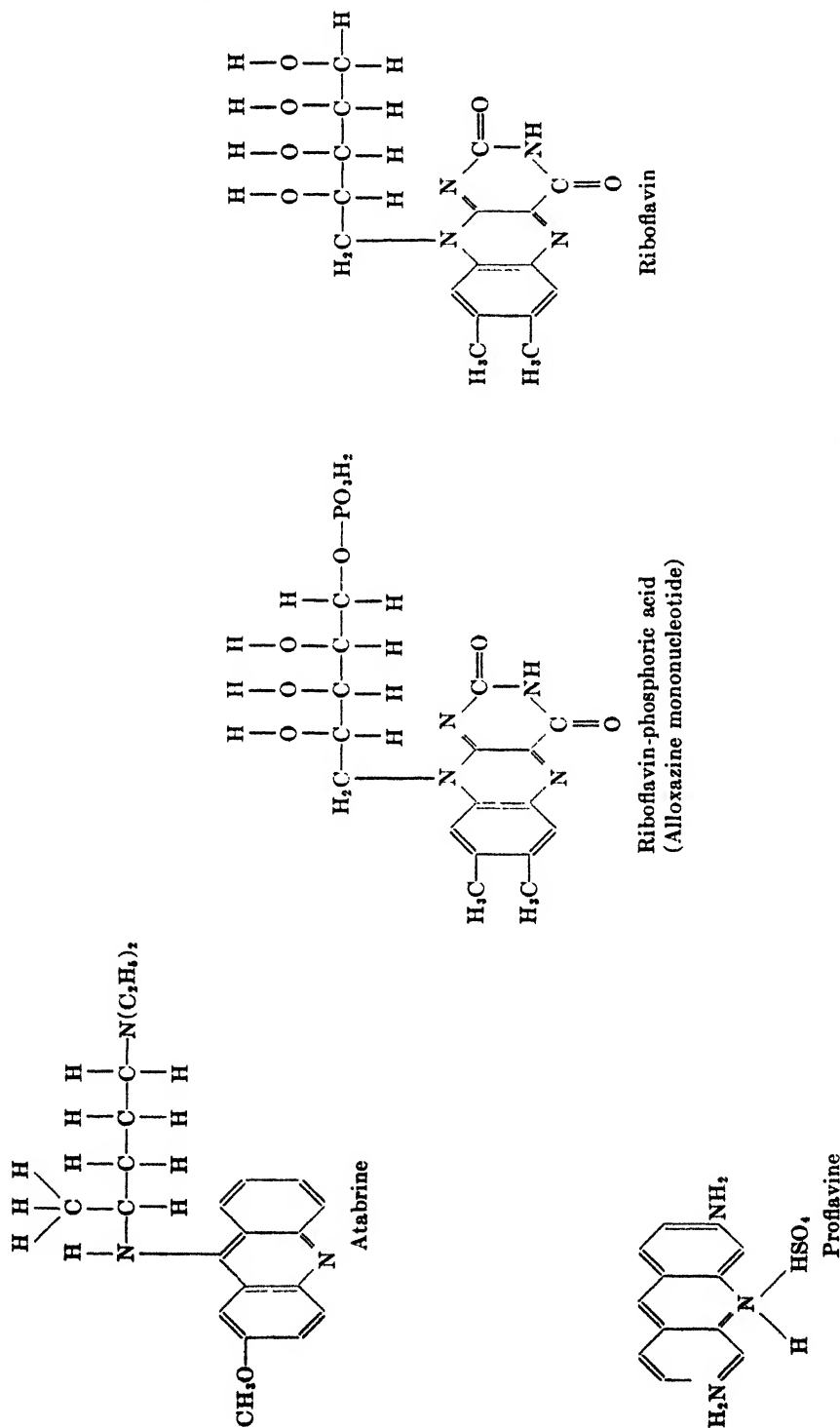


FIG. 3. RIBOFLAVIN AND ITS CORRESPONDING COENZYME

Riboflavin, atabrine, the acridine ring, adenine, and adenosine are suggested as substituents on the N<sup>1</sup> nitrogen of sulfanilamide for new sulfonamides of theoretical interest and possible practical value.

Steps in the enzymatic oxidation of glucose thus represent critical sources of the energy required for cell division, which may be vulnerable to the action of chemotherapeutic agents. In view of the great clinical success of the sulfonamides in which pyridine, thiazole, and pyrimidine are substituents on the N<sup>1</sup> nitrogen of sulfanilamide, and of the relationships schematized, it would be extremely interesting to have series of sulfonamides in which alloxazine, atabrine, or acridine are substituents on the N<sup>1</sup> nitrogen of sulfanilamide<sup>4</sup> (figure 3). Because of the critical role in glucose oxidation of phosphorylation, mediated by the adenosine phosphoric acid enzymes, it would be exceedingly interesting also to have series of sulfonamides in which adenine and adenosine are similarly coupled with sulfanilamide.

The oxidations of glucose into its intermediate products, however, may proceed by several different pathways (for details see Potter, 1944), and for the further transformation of pyruvic acid seventeen different reaction paths are already known. *Multiple simultaneous inhibitions*, rather than single inhibitions, may then be necessary for the suppression of the intracellular multiplication of a disease agent and therefore may afford the key to successful chemotherapy. These considerations afford a rationale for systematic study of the *synergistic action of various chemotherapeutic agents*: sulfonamides, penicillin, atabrine, quinine, arsenicals, diamidines, and heavy metal compounds. Instances are already on record of two chemotherapeutic agents together accomplishing what neither one can do alone (Ungar, 1943; Bigger, 1944; Soo-Hoo and Schnitzer, 1944; Harned, Miller, Wiener, and Watts, 1944; T'ung, 1944; Kirby, 1944; Hobby and Dawson, 1945; Roblin *et al.*, 1945). However, since synergistic action producing multiple inhibitions on host cell enzymes essential to intracellular multiplication of the parasite might be also injurious to the host, any application of this type of study to man, pending thorough investigation *in vitro* and in animals, would seem premature and dangerous.

The investigation of bacterial respiratory and other metabolic systems as a basis for a rational chemotherapy will bring our discipline into close association with studies of nutrition, normal and abnormal growth, and the physiology of muscular, nervous, and glandular function. The mature synthesis which will eventually result will surely make manifold return in understanding of fundamental life processes and in applications to human well-being.

<sup>4</sup> In the acridine derivatives listed by Northey (1940) substitution was on the N<sup>4</sup> nitrogen.

Pyrazine, which forms the central ring of riboflavin, has been used in the pyrazine analog of sulfapyridine (Ellingson, 1941). Sulfapyrazine is reported to be of the same order of efficacy as sulfadiazine (Robinson, Siegel, and Graessle, 1943).

After the above was written, Dr. E. H. Northey kindly furnished the writer references recording the synthesis of acridine derivatives of sulfanilamide (Ganapathi and Nandi, 1940; Das-Gupta, 1941; Coggeshall and Maier, 1942). Ganapathi (1940) writes:

"The compound, 2-N<sup>1</sup>-sulphanilamidothiazol, which has since been reported by Fossbinder and Walter [sulfathiazole] and 2:8-sulphanilamidoacridine [italics the author's] possess striking protective action in experimental streptococcal and pneumococcal infections in mice."

Ganapathi (1941) has also recorded the synthesis of 7-N<sup>1</sup>-sulphanilamidoalloxazine which he reported to be therapeutically inactive in mice. It does not follow of course that among related derivatives an active one might not be found.

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# THE GROWTH OF AEROBIC THERMOPHILIC BACTERIA

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The thermophilic microbes are usually defined as organisms capable of growing at high temperatures. Such a definition does not imply that life at high temperature is actually linked with a modified physiology of nutrition, reproduction, etc. The microbes adapt themselves not only to high temperatures, but also to the physico-chemical peculiarities of their habitat at these temperatures. Such an ecological approach suggests that the thermophiles represent a rather isolated group of microbes differing somewhat in their biology from mesophilic bacteria.

It was pointed out in previous publications that the biochemical activity of thermophilic bacteria is extremely high (Imšenecki, 1941a, 1941b). The leading role among the causes underlying this increased activity belongs to rapid growth of thermophiles, and for this reason the present study deals with the peculiarities of reproduction of the sporulating aerobic thermophilic bacteria. Some parallel experiments were also conducted with mesophilic bacteria.

## *The Behavior of Thermophilic Bacteria at Relatively Low Temperatures*

Thermophilic bacteria very often occur at temperatures lower than those necessary for their growth. Hence it is commonly held that under such conditions the thermophiles are in a resting state (mostly in the spore state) and do not participate in the mineralization of organic matter. Revival of the thermophiles and the beginning of their active life is caused by a rise of temperature as linked, e.g., with heating of the superficial layers of the soil by sunlight or with self-heating of the disintegrating organic matter.

Yet, inactivity of thermophiles at relatively low temperatures is sometimes denied. It was shown in a number of studies that the thermophiles are able to propagate not only at high but also at low temperatures, such as 20 to 30 C (Koch and Hoffmann, 1911; Wallace, 1924; Tanner and Wallace, 1925; Hansen, 1933). It will be noted that growth of the thermophilic bacteria at these low temperatures proceeds so slowly that the division of one cell takes 6 hours (Hansen, 1933). Hence the development of the thermophiles at low temperatures has been overlooked. Thus, the thermophiles live and reproduce at relatively low temperatures and participate in the circulation of matter.

Attention was therefore paid, in the present study, to the capacity of thermophilic bacteria to grow at different temperatures. By inoculating meat peptone agar plates with the contents of the intestine of various animals, soil, or slime, it is easy to cultivate colonies at 60 C. However, the overwhelming part of the colonies belongs to species which in pure cultures grow rapidly not only at 60 C but also at such low temperatures as 25 to 30 C. The optimum temperature

range for growth of these species is 40 to 50 C. Hence the great majority of the forms cultivated at 60 C belong not to the truly thermophilic but to thermo-tolerant species.

As to true thermophiles, i.e., species whose optimal range of growth lies between 55 to 65 C, one may distinguish two groups of organisms. One of them develops at 60 C but does not show any growth during several days at 28 to 30 C. Following the general biological nomenclature one may call them stenothermal thermophiles.

The representatives of the other group of thermophilic bacteria also show a growth optimum at about 60 C, but a slight growth may also be seen at such low temperatures as 28 to 30 C, that is to say, these are eurithermal thermophiles. It is important in determining the temperature range of growth that tests also be made for visible growth on the surface of agar at relatively low temperatures. However, in liquid media or in the condensation water in agar test tubes such apparently negative tube cultures may show slow growth, which brings about an

TABLE 1

*Cultivation of thermophilic bacteria at 28 to 30 C*

(The figures correspond to the number of cells in thousands per 1 ml as found by direct count)

EXP. NO.	BACILLUS SP. (STRAIN 1) EURITHERMAL THERMOPHILE		B. DIASTATICUS STENOTHERMAL THERMOPHILE	
	Initial cell number	After 5 days	Initial cell number	After 5 days
1	510	50,064	6,115	2,675
2	2,675	35,287	1,656	2,038
3			4,713	5,350
Avg. . . . .	1,598	42,676	4,161	3,354

opacity of the fluid. This may be illustrated by the protocols of two experiments. In the first of these the culture of the thermophile, *Bacillus* sp. (strain 1), was inoculated on meat peptone broth of 1-cm depth. No perceptible growth was noted at 28 to 30 C on meat peptone agar by the naked eye during 3 to 4 days. In the second experiment the development of the thermophile *Bacillus diastaticus* nov. sp., which intensely hydrolyzes starch, was studied. The description of this species has been given elsewhere (Imšenecki *et al.*, 1942). *Bacillus diastaticus* was cultivated on 5 per cent potato decoction with 0.1 per cent chalk, also in a thin layer of liquid. In both experiments the number of cells was determined in the contents of the flasks immediately after inoculation and after 5 days' incubation in a thermostat at 28 to 30 C. The method of direct count of stained bacteria was used.

The figures of table 1 show that in 5 days the number of cells of *Bacillus* sp. increased by approximately 27 times. Hence, although slowly, the bacteria do reproduce, and the species in question may be classified as an eurithermal thermophile.

As to the amylolytic bacterium, *Bacillus diastaticus*, which at 60 C develops vigorously on the potato decoction, no growth whatever was noted in the same medium during 5 days' cultivation at 28 to 30 C. Such a behavior is characteristic of stenothermal thermophiles, which occur in nature much less frequently than the representatives of the first group.

The species not developing at low temperatures, i.e., stenothermal thermophiles, include also *Bacillus cellulosa-dissolvens*. A pure culture of this species rapidly fermented cellulose at high temperatures but not at relatively low temperatures. This is shown by the following experiment. In the midst of fermentation the flask with the decomposing cellulose was removed from the thermostat and the fermentation products were determined in the culture fluid. Then the flask was kept at 20 C during 10 days and the analyses repeated. In both experiments the same amount of hydrolytic products of cellulose and of volatile acids was found in the medium. Hence at relatively low temperatures cellulose is not fermented. Some investigators who dealt only with stenothermal species concluded that at a relatively low temperature no growth occurs. In studying eurithermal species one could make the mistake of attributing to all thermophiles the capacity of growth at low temperatures. In fact, however, there exist in nature both groups of thermophiles.

#### *Growth of Thermophilic Bacteria at High Temperatures*

Experiments were conducted on *Bacillus diastaticus* and *Bacillus* sp. (strain 1) to get an idea as to the rate of growth of the thermophiles at 60 C. Erlenmeyer flasks of 250-ml capacity were filled with thin layers (1 cm) of the liquid medium previously mentioned. The flasks were inoculated with 17-hour cultures on liquid media. Cell counts were made immediately after inoculation and in cultures at different ages. Prior to inoculation, the cultures were diluted in a liquid nutrient medium brought to 60 C. In the experiments with *Bacillus* sp. the number of cells was determined both by direct cell count and by plating on meat peptone agar. To secure cell counts in young cultures the flasks were inoculated with relatively large doses of microbes. In *Bacillus diastaticus* the cell count was made by plating in petri dishes on solid nutrient medium of the following composition: 20 per cent potato decoction, 100 ml; peptone, 0.5 g; chalk, 0.1 g; agar, 2.0 g. The results of these experiments are summarized in tables 2 and 3.

It will be seen that the number of cells of *Bacillus* sp. and *Bacillus diastaticus* rapidly increases and very soon reaches its maximum. Reproduction of thermophilic bacteria begins almost at once after inoculation of the culture. The initial stationary phase is either absent or is so short that our methods did not reveal it. Growth is more rapid in the thermophiles than in the case of mesophiles, that is, the generation time is shorter. In the thermophilic bacteria there occurs a more rapid diminution of the number of cells in the culture, and hence the stationary phase is followed by a rapid decline. Mesophiles are characterized by a slower diminution of the number of bacteria.

Of considerable interest is the comparison of the results obtained with *Bacillus*

sp. (strain 1) by determining the number of cells by cell count and inoculation (table 2). By comparing the direct count and the plate count it is possible to elucidate the correlation between viable and dead cells and those which do not germinate on the surface of solid media. It will be seen that the number of cells obtained by direct count increases more rapidly than that obtained by

TABLE 2

*Growth of the thermophilic bacteria (Bacillus sp., strain 1)*

(The figures correspond to the number of cells in thousands per 1 ml as found by direct count)

EXP. NO.	IMMEDIATELY AFTER INOCULATION		AGE OF CULTURE									
			4 hours		6 hours		17 hours		24 hours		36 hours	
	Inoculated in plates	Direct count	Inoculated in plates	Direct count	Inoculated in plates	Direct count	Inoculated in plates	Direct count	Inoculated in plates	Direct count	Inoculated in plates	Direct count
1	8,056	3,185	11,176	43,567	27,296	74,396						
2	5,160	1,401	17,720	7,134	81,760	38,090						
3	8,240	764	29,340	20,255	45,260	35,160						
4	2,233	11,720					258,200	287,137	101,500	210,703		
5	470	6,115					289,100	332,233	61,300	339,622		
6	5,760	5,605					145,900	446,629	111,800	273,634		
7	7,470	8,663					195,000	500,897	204,800	329,431		
8	1,031	4,968									12,040	19,873
9	484	2,675									10,160	42,803
10	709	4,104									9,840	36,434
Avg.	1,895	4,274	19,412	23,652	51,439	49,215	222,050	391,724	119,850	288,349	10,680	33,037

TABLE 3

*Growth of the thermophilic amylolytic bacteria (Bacillus diastaticus)*

(The figures correspond to the number of cells in thousands per 1 ml)

EXP. NO.	IMMEDIATELY AFTER INOCULATION (CONTROL)	AGE OF CULTURE		
		24 hours	48 hours	72 hours
1	2.8	20,400	1,490	370
2	13.6	24,300	830	1,120
3	15.2	7,000	2,900	59
4	7.4	3,800	600	240
Avg. ....	9.75	13,875	1,455	447

plating. This discrepancy reaches a maximum at the apex of the curve (maximum stationary phase), which suggests that the number of dying cells rises continuously. It will also be noted that if the dead bacteria did not rapidly disintegrate, they would have been counted, even though reproduction of the cells had stopped. Microscopic examination of cultures of different ages completely confirms this view. In relatively young thermophile cultures there always occur

poorly staining ghosts of cells, as well as cells with granular contents. The morphological analysis reveals extremely rapid aging of the culture and early disintegration of the cells. Dead cells are usually numerous in 24-hour cultures, in which amorphous granular debris accumulates.

Thus, not only are thermophilic bacteria characterized by extremely rapid reproduction, but also no less characteristic is the rapid aging and dying of the cells. The whole rate of life of thermophiles is increased; the faster the division of the cells, the sooner their death. In spite of the fast propagation of the thermophiles, the total number of cells in the cultures without aeration is relatively low. For *Bacillus* sp. the maximum number of cells per 1 ml of culture amounts (direct count) to about 392 million; for *Bacillus diastaticus* to only 14 million. It seemed therefore worth while to determine the maximum number of cells in the cultures of mesophilic sporulating bacteria. For this purpose cultures of *Bacillus malabarensis* were taken, as well as *Bacillus megatherium* and *Bacillus pseudoanthracis*, because our strains of these species do not form a film which impedes cell count. The mesophiles were cultivated in flasks with meat

TABLE 4

*Number of cells in the cultures of mesophilic bacteria*

(The figures correspond to the number of bacteria in thousands in 1 ml)

B. MALABARENSIS		B. MEGATHERIUM		B. PSEUDOANTHRACIS	
Initial number	After 48 hr	Initial number	After 48 hr	Initial number	After 48 hr
17,325	2,430,601	19,109	1,959,258	21,147	2,140,152
8,663	830,583	21,911	1,643,331	10,446	1,457,342
15,542	1,070,076	23,185	1,757,982		
Avg 13,843	1,443,753	20,510	1,801,295	15,797	1,798,747

peptone broth; in other respects the methods of study were similar to those previously described. It was shown by preliminary experiments that the number of cells in the cultures of all three mesophile species gradually increases, attaining a maximum in 48-hour cultures.

The results of the experiments are summarized in table 4, which shows that in mesophiles the number of bacteria in the culture is very great, viz., from 1,443 to 1,801 million, on the average. Thus, in thermophile cultures the absolute number of cells is considerably less than in the mesophiles. This difference is probably less than appears to be the case since in the thermophile cultures autolysis and decomposition of the cells may have already begun, whereas in mesophiles autolysis begins much later. However, this discrepancy cannot be great as fewer cells are seen in very young thermophile cultures in which disintegration of the cells has not as yet begun.

A suggestion was made, on ecological grounds, to account for the difference in the number of cells in the cultures of thermophilic and mesophilic bacteria. The amount of dissolved oxygen in water is known to decrease with an increase in temperature, and it is logical to expect that one restricting factor in the growth

of thermophiles is insufficient aeration of the medium. This seemed quite plausible as observations on thermophilic bacteria made during many years show that aerobic thermophiles reproduce well only in a thin layer of liquid medium. In flasks with a high column of fluid, development of the thermophile cultures is greatly impeded. The following experiments were aimed at an experimental verification of this suggestion.

*Effect of Aeration on the Rate of Growth*

The following methods were used. Glass cylinders of 50-ml capacity were filled with 30 ml of the liquid medium previously mentioned. A rubber tube with minute orifices in its lower end was immersed in the medium. The tube was passed through the cotton stopper of the cylinder. The lower end of the tube was hermetically closed with a glass rod, whereas the upper end was connected with a glass tube through which sterile air heated to 60 C was passed. The medium within the cylinders was inoculated with 2 ml of an 18-hour culture grown in a liquid medium at 60 C. Immediately after inoculation the number

TABLE 5

*Effect of aeration of culture on the growth of thermophilic bacteria*  
(The figures correspond to the number of bacteria in thousands in 1 ml)

EXP. NO.	BACILLUS DIASTATICUS			EXP. NO.	BACILLUS SP. (STRAIN 1)		
	Initial number	After 6 hr	After 8 hr		Initial number	After 6 hr	After 8 hr
1	16,306	324,699	737,079	1	10,446	1,283,072	2,225,758
2	8,408	236,436	438,731	2	25,478	1,494,794	3,020,672
				3	6,879	1,291,735	1,635,688
Avg..	12,357	330,068	587,905		14,268	1,359,200	2,294,039

of cells in the cylinders was determined by direct count, and the cylinders were placed in a thermostat at 60 C. Air was passed through the medium throughout the experiment. Cell counts were made of the culture after 6 and 8 hours of aeration.

It is seen from the data of table 5 that aeration appreciably accelerates the reproduction of thermophilic bacteria. Under these conditions the number of cells per 1 ml of culture of *Bacillus diastaticus* attains 587 million after 8 hours, and 2,294 million in the case of *Bacillus* sp. A comparison of these figures with those obtained in the nonaerated cultures (thin layer) shows that aeration greatly increases the number of cells.

Thus, by means of aeration it is possible to shift appreciably the growth curve of thermophilic bacteria and to obtain the maximal number of cells in less time. In cultivating *Bacillus diastaticus* in large volumes of liquid for the production of amylase preparations, the cultivation time of the thermophiles can be reduced to a minimum. Thus, upon inoculation of the medium with 5 to 10 per cent of culture, and with more perfect aeration than in the laboratory experiments,

reproduction of the bacillus and the accumulation of amylase in the medium attain a maximum after 4 to 5 hours.

#### DISCUSSION

As a result of adaptation to high temperatures there have arisen in nature thermophilic microbes among which occur species the temperature optimum of which surpasses the temperature causing death of the mesophiles. Such an adaptation can be simulated under laboratory conditions; by means of a gradual increase of temperature it is possible to obtain forms adapted to higher temperatures (Dallinger, 1887; Dieudonné, 1894; Tsiklinsky, 1899). However, an appreciable change of the temperature maximum requires a considerable span of time. Thus, the sudden formation of thermophiles, which is considered possible (Lieske, 1921; Kluyver and Baars, 1932), seems very doubtful.

The thermophilic bacteria present an isolated group of microbes of secondary origin which have adapted themselves to life in surroundings with a high temperature. This group may include thermophilic strains of mesophiles, as is the case with bacteria fermenting cellulose, as well as species which do not appear to have parallel forms among the mesophiles. Some thermophiles have lost the capacity to grow, even slowly, at relatively low temperatures, whereas others have retained this capacity.

Life at high temperatures calls forth a change in the whole biology of the microbes. Thus, characteristic of the thermophiles is rapid growth at high temperatures. Even in the slowly multiplying actinomycetes, thermophilic strains show rapid growth (Imšenecki and Avdievich, 1944). Vigorous reproduction of the thermophiles is followed by rapid aging, death, and disintegration of the cells. These bacteria obey the general law that the more rapid the reproduction, the shorter the survival time. Both phenomena, intense growth and rapid death, occur at the optimal temperature (for reproduction) of 55 to 65 C, but this temperature is not optimal for the preservation of the species.

For practical purposes, rapid reproduction of bacteria is essential, whether or not there occur degenerative changes and mass death of the cells. In those cases in which rapid reproduction of the bacteria and accumulation of enzymes in the medium are required, the thermophilic microbes have some advantages. It is probably the early disintegration and autolysis of the thermophilic microbes, with the release of the intracellular enzymes, that account for the more rapid accumulation of the enzymes in the medium with the thermophiles than occurs with the mesophiles.

#### SUMMARY

Two groups may be distinguished among the thermophilic bacteria: stenothermal thermophiles not developing at relatively low temperatures, and euri-thermal thermophiles growing at these temperatures.

Rapid reproduction at high temperatures is one of the most characteristic peculiarities of thermophiles. Their growth curve differs from that of mesophiles.



Following rapid reproduction the cells of thermophiles soon die away, undergoing autolysis and disintegration. Accelerated aging of the culture is also one of the thermophile peculiarities.

Aeration of the culture appreciably accelerates reproduction of the aerobic thermophilic bacteria.

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# THE ANTIGENIC STRUCTURE AND SPECIFICITY OF LUMINOUS BACTERIA

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Although the immunological reactions of the luminous bacteria have received little study, they justify investigation from three points of view: first, the nature of their antigenic structure; second, the extent of species specificity in the reactions concerned; and, finally, the significance of antigenic components in the luminescent system.

This study has included agglutination, agglutinin absorption, and precipitation tests in relation to whole cells as well as the filtrates obtained from cytolytic products. These procedures were applied to all the well-authenticated species available. Previous studies in which immunological techniques were employed have dealt almost exclusively with whole cells and with only very restricted groups of species or strains (Ballner, 1907; Ninomya, 1924; Meissner, 1926; Majima, 1931). Varying degrees of specificity and the occurrence of group reactions have been noted in these limited studies. Johnson (1941) cytolized the cells of two marine species in distilled water, and found a specific agglutination of the "ghosts" as well as the normal cells, and a specific precipitation of Berkefeld filtrates of the cytolyzates.

The phenomena associated with the cytolysis of these organisms have been extensively investigated from the point of view of physiological activity (Harvey, 1915; Hill, 1929; Korr, 1935a, 1935b; Johnson and Harvey, 1937, 1938) as well as the fine structure of the cells (Johnson, Zworykin, and Warren, 1943). In the report by Harvey and Deitrich (1930), however, the production of antibodies against the oxidative enzyme (luciferase) concerned in the luminescence of extracts of the invertebrate animal *Cypridina* is described.

Although a decrease in light intensity of luminous bacteria has been observed in the presence of immune serum (Ninomya, 1924), the results thus far reported have evidently been due entirely to the agglutination of the cells. A loss of a completely adequate gaseous respiratory exchange occurs concomitantly with agglutination. Furthermore, as observed in the present study, easy confirmation of this explanation may be obtained by observing the reappearance of the original light intensity upon vigorously shaking the agglutinated cells.

## MATERIALS AND METHODS

The species used in these studies are *Vibrio albensis* (Lehmann and Neumann, 1901)<sup>2</sup>; *Vibrio phosphorescens*<sup>3</sup>; *Achromobacter harveyi* (Johnson and Shunk,

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<sup>2</sup> Cultures of these species, under the generic name *Photobacterium*, were obtained in 1939 from the Delft Collection, through the kindness of Professor A. J. Kluyver.

<sup>3</sup> Original culture was kindly supplied by Professor M. H. Soule.

1936); *Achromobacter fischeri* (Beij.) Bergey *et al.* (Bergey, 1934; Johnson and Shunk, 1936); *Photobacterium phosphoreum* (Cohn) Beij. (1912, 1916)<sup>2</sup>; *Bacillus pierantonii* (Zirpolo, 1918)<sup>2</sup>; *Bacillus sepiae* (Zirpolo, 1917)<sup>2</sup>; and *Photobacterium splendidum* (Beijerinck, 1916)<sup>2</sup>. Of the eight species, six are marine forms and were cultivated on 3 per cent NaCl beef infusion agar. The remaining two, *Vibrio albensis* and *Vibrio phosphorescens*, are "fresh water" species and were cultivated on the same medium as the marine species, except that the medium contained only 0.9 per cent NaCl. The medium was adjusted to pH 7.3 and sterilized at 15 pounds' pressure for 15 minutes. *P. phosphoreum*, a psychrophilic type, was cultivated at 15 C., but the others were incubated at 25 C.

These organisms are as authentic as could be obtained. Their possible identity with organisms described in the early literature (Beijerinck, 1889, 1890, 1916; Dahlgren, 1915; Gorham, 1903; Lehmann and Neumann, 1901; Mangold, 1910; Migula, 1897, 1900; Molisch, 1912) cannot be fully established in all cases. The literature concerning the luminous species, especially in regard to nomenclature, is unusually confusing.<sup>4</sup> The problem is perhaps somewhat further complicated by the occurrence of variants (cf. Beijerinck, 1912; Doudoroff, 1938; Giese, 1943). The characteristics of the species used in this study have, therefore, been reinvestigated and are summarized in tables 1, 2, and 3.

The organisms that have been previously studied are *Vibrio rumpel* (Ballner, 1907); *Vibrio pierantonii*, *Coccobacillus pierantonii*, *Bacillus sepiae* (Meissner, 1926); and *Vibrio pierantonii*, *Coccobacillus pierantonii*, *Vibrio euprime*, *Micrococcus sepiola*, *Coccobacillus tolega*, *Vibrio yasakii* (Majima, 1931).

#### EXPERIMENTAL

##### *Agglutination Reactions*

Luminous cultures were suspended in sterile blanks of the appropriate salt concentration. Rabbits were then injected in duplicate with each organism

<sup>4</sup> For example, Fischer, in 1888, discovered what he termed an "*Einheimischer Leucht-bacillus*" in the water of the Kiel harbor. In 1889 Beijerinck included all luminous bacteria in the special genus *Photobacterium* and renamed Fischer's bacterium, *Photobacterium fischeri*. Katz described an organism which he designated as *Bacillus argenteo-phosphorescens* I. Migula (1897, 1900) maintained that this organism was a variety of *Photobacterium fischeri* (Beij.) and classified it as such in his *System der Bakterien* (1900). However, Katz had previously isolated *Bacillus argenteo-phosphorescens* which Migula (1900) assumed to be a synonym of *Bacillus argenteo-phosphorescens* I. In 1897 Migula rejected Beijerinck's *Photobacterium* genus for this organism and included it in the genus *Bacillus*, i.e., *Bacillus fischeri* (Beij., Mig.). Flügge (1896) reclassified this organism under *Bacillus phosphorescens-indigenus* Kruse. Further synonyms have been given: *Vibrio fischeri* (Beij.) (Lehmann and Neumann, 1901), *Achromobacter fischeri* (Beij.) Bergey *et al.* (1930).

A further illustration of the susceptibility of these organisms to possible errors in classification appears in Bergey's *Manual of Determinative Bacteriology*, 5th edition, 1939. Under the description of *Pseudomonas phosphorescens* (Fischer) Bergey, the following organisms are cited as synonyms: *Photobacterium phosphorescens* (Beijerinck, 1890); *Bacillus phosphorescens* (Fischer, 1888); and *Bacterium phosphorescens* (Migula, 1900). However, when the literature is consulted, it is apparent that *Bacillus phosphorescens* is evidently not synonymous with any of the other organisms mentioned above. *Photobacterium phosphorescens*, as Bergey indicates, is synonymous with *Bacterium phosphorescens*, whereas *Bacillus phosphorescens* is a distinct species (Migula, 1897).

TABLE 1

*Major characteristics of eight species of luminous bacteria*

ORGANISM	MORPHOLOGY	MOTILITY	FLAGELLA (ELECTRON MICROSCOPE)	CAPSULE*	OPTIMUM NaCl CON- CENTRATION	GELATIN (LIQUEFAC- TION)	REDUCTION OF NITRATE TO NITRITE	INDOLE†	BLOOD AGAR			STARCH DIGESTION	H <sub>2</sub> S
									Growth	Luminescence	Hemolysis‡		
Fresh-water species—0.9% NaCl included in media													
<i>V. albensis</i> ..	Vibrio single and pairs 1.2 -2.1 μ gram-	+	Mono- tri- chate	-	0.9	++	+	+	++	+	Beta	+	-
<i>V. phospho- rescens</i> . .	Vibrio single and pairs 0.8 -2.5 μ gram-	+	Mono- tri- chate	-	0.9	++++	+	+	++	+	Al- pha	+	-
Marine species—3% NaCl													
<i>A. harveyi</i>	Rods—str., curved single and pairs. Ends: pointed, rounded, 1.2- 2.3 μ gram-	+	Peri- or lopho- tri- chate	-	3	+++	+	+	++	+	-	+	+
<i>A. fischeri</i>	Rods—str., curved single and pairs. Ends: pointed, rounded, 0.9- 1.8 μ gram-	+	Peri- or lopho- tri- chate	-	3	+	+	-	++	+	-	-	+
<i>P. phospho- reum</i>	Oval cocci single and pairs 1.2-2.2 μ gram±	-	-	+	3	-	+	-	+	+	-	-	-
<i>B. pierantonii</i>	Rods—str., curved rounded ends 0.9-2.2 μ gram	+	?	-	3	+	+	-	++	+	Al- pha	-	-
<i>B. sepiæ</i> . . . .	Rods—str., sin- gle, pairs, chains, 1.2- 2.6 μ gram-	+	Peri- or lopho- tri- chate	-	3	+++	+	+	++	+	Al- pha	+	+
<i>P. splendidum</i>	Rods—str. sin- gle and pairs 1.0-2.8 μ gram	+	Peri- or lopho tri- chate	-	3	+++	+	+	++	+	Al- pha	+	-

\* Welch's method.

† Gore's method.

‡ The hemolysis results are unaccountably variable.

TABLE 2  
Characteristics of luminous bacteria (con.)

ORGANISM	GLUCOSE		LACTOSE		SUCROSE		KOSER'S CITRATE MEDIUM		GROWTH IN PEPTONE BROTH (72 hours)				
	Acid	Gas	Acid	Gas	Acid	Gas	Growth	Luminescence	8-10 C	15 C	20 C	25 C	37 C
Fresh-water species—0.9% NaCl													
<i>V. albensis</i> .....	+	—	—	—	+	—	+	+	—	+	++	+++	++++
<i>V. phosphorescens</i> .....	+	—	—	—	+	—	+	++	—	++	++++	++++	++++
Marine species—3% NaCl													
<i>A. harveyi</i> .....	+	—	—	—	+	—	++++	++++	—	+++	++++	++++	++++
<i>A. fischeri</i> .....	+	—	—	—	—	—	—	—	—	+++	+++	+++	—
<i>P. phosphoreum</i> .....	+	+	—	—	+	—	+	++	++	++++	++	+	—
<i>B. pierantonii</i> ...	+	—	—	—	+	—	++	±	—	+++	+++	+++	++++
<i>B. sepiae</i> .....	+	—	—	—	+	—	+	—	—	+++	++++	++++	++++
<i>P. splendidum</i> ...	+	—	—	—	—	—	++	—	—	++++	++++	++++	++++

TABLE 3  
Characteristics of luminous bacteria (cont.)

ORGANISM	LUMINESCENCE (72 HOURS)					PERSISTENCE OF LUMINESCENCE (DAYS)					PER CENT NaCl TOLERANCE (PEPTONE BROTH)			
											Growth		Luminescence	
	8-10 C	15 C	20 C	25 C	37 C	8-10 C	15 C	20 C	25 C	37 C	Min	Max	Min	Max
Fresh-water species—0.9% NaCl														
<i>V. albensis</i> ....	—	++	++	+++	+++	—	11	19	16	11	0	4.1	0	1.2
<i>V. phosphorescens</i> .....	—	—	++++	++++	+++	—	15	25	26	6	0	4.5	0	2.1
Marine species—3% NaCl														
<i>A. harveyi</i> ....	—	±	++	++	—*	—	33	32	34	2	0.6	8.7	0.6	6.9
<i>A. fischeri</i> ....	—	++	++++	++++	—	—	48	46	43	—	0.6	6.9	1.5	6.0
<i>P. phosphoreum</i> .....	++	++++	++++	++	—	71	59	43	23	—	0.9	5.4	0.9	5.1
<i>B. pierantonii</i>	—	+	+	++	++	—	7	17	12	—	0.3	6.9	2.4	4.1
<i>B. sepiae</i> .....	—	—	++	++++	—	—	17	26	19	—	0.6	8.7	6.0	7.8
<i>P. splendidum</i>	—	++	+++	+++	—*	—	32	18	13	2	0.3	9.0	0.6	6.9

\* Luminescence appears at an earlier incubation period.

over a period of 5 weeks. The animals received 1-ml suspensions of freshly prepared luminous 18- to 24-hour cultures at 48-hour intervals over a 3-week period. A rest period of 7 days followed, after which they received a 2-ml intra-

TABLE 4

Agglutination and cross agglutination of eight species of luminous bacteria with rabbit antisera prepared from the following luminous cell antigens: *V. albensis*, *A. fischeri*, *A. harveyi*, *V. phosphorescens*, *P. phosphoreum*, *B. pierantonii*, *B. sepiae*, and *P. splendidum*

(The table includes all cross agglutinations that were found)

ANTIGEN	DILUTION OF ANTISERUM (FINAL DILUTION)												CON- TROL
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	1:20480	1:40960	
A. Anti-albensis													
<i>V. albensis</i> .	4	4	4	4	4	4	3	2	2	1	1	0	0
<i>V. phosphores- cens</i> . . . . .	2	2	2	2	2	1	1	1	0	0	0	0	0
B. Anti-phosphorescens													
<i>V. phosphores- cens</i> . . . . .	4	4	4	4	4	4	4	2	1	1	0	0	0
<i>V. albensis</i> .	4	4	4	4	4	4	3	2	1	0	0	0	0
C. Anti-harveyi													
<i>A. harveyi</i> .	4	4	4	4	3	2	2	1	0	0	0	0	0
<i>P. splendidum</i> .	2	2	1	1	1	0	0	0	0	0	0	0	0
D. Anti-splendidum													
<i>P. splendidum</i>	4	4	4	4	3	2	2	2	1	1	0	0	0
<i>B. sepiae</i> . . . .	3	1	1	1	±	0	0	0	0	0	0	0	0
E. Anti-sepiae													
<i>B. sepiae</i> . . . . .	4	4	4	4	3	2	1	1	0	0	0	0	0
F. Anti-fischeri													
<i>A. fischeri</i> . . . . .	4	4	4	4	4	4	3	2	1	0	0	0	0
G. Anti-phosphoreum													
<i>P. phosphoreum</i>	4	4	4	4	3	3	2	1	1	0	0	0	0
H. Anti-pierantonii													
<i>B. pierantonii</i> .	4	4	4	4	4	4	4	3	2	2	1	0	0

0 indicates no agglutination; 1, trace; 2, moderate; 3, almost complete; and 4, complete agglutination.

venous injection. After a second rest period of similar length, the rabbits were bled by heart puncture. The serum of each rabbit was tested at definite intervals during the period of injections for agglutination titer.

Antisera were set up in dilutions ranging from 1:20 to 1:40,960, using the appropriate salt concentration for the dilution of the antisera. To each tube

and the control, 0.5 ml of the homologous antigens were added. At the same time, cross agglutination reactions were carried out with each of the 8 species. All tubes were incubated in a 25 C water bath for 4 hours, after which they were stored overnight in an icebox and read the following morning.

The study of the agglutination reactions was complicated by the autoagglutination of several species. This was especially true of *V. albensis* and *A. fischeri*. However, this was remedied by centrifuging and washing the cells thoroughly.

An examination of the data reveals that *A. fischeri*, *P. phosphoreum*, *B. pierantonii*, and *B. sepiac* are antigenically specific. Cross agglutination occurs between *V. albensis* and *V. phosphorescens*, between *A. harveyi* and *P. splendidum*, and between *P. splendidum* and *B. sepiac*.

The cross agglutination of *A. harveyi* and *P. splendidum* has been confirmed by Johnson (unpublished). However, since only *A. harveyi* antiserum will react with *P. splendidum* and not vice versa, the relationship is obviously not of a reciprocal nature. This is also true in the case of *P. splendidum* antiserum and *B. sepiac* antigen. On the other hand, the *V. albensis*-*V. phosphorescens* cross reaction occurs reciprocally with these two organisms. It is interesting to note that although anti-*V. albensis* serum cross-agglutinates *V. phosphorescens*, the homologous strain is agglutinated to a higher titer. When anti-*V. phosphorescens* serum is used, an equal titer occurs with both antigens.

#### Agglutinin Absorption

For agglutinin absorption tests, heavy suspensions of cells were harvested in the appropriate sodium chloride solution, centrifugalized, and washed once. Absorptions were carried out in a final 1 to 20 dilution of serum. The tubes were well shaken and then incubated at 25 C for 3 hours. This was followed by strong centrifuging, i.e., until the supernatant was practically clear. This procedure was repeated with the supernatant serum and a fresh suspension of cells. Following the second incubation period, the tubes were left in the icebox overnight before centrifuging.

The results of the agglutinin absorption tests, which are shown in table 5, confirm the results of the straight agglutination reactions. It is evident that *P. splendidum* almost completely absorbs its corresponding antibody from anti-*A. harveyi* serum without appreciably changing the agglutination titer of the serum with respect to *A. harveyi*. Similarly, *B. sepiac* absorbs its antibody from anti-*P. splendidum* serum without altering the titer of the serum for *P. splendidum*.

Cross absorption experiments were set up for both *V. albensis* and *V. phosphorescens*. Although it was not possible<sup>5</sup> for *V. albensis* to absorb the entire *V. phosphorescens* agglutinin from the anti-*V. phosphorescens* serum, a good fraction of the heterologous agglutinin was absorbed. On the other hand, the ab-

<sup>5</sup> In view of the close antigenic relationship between *V. albensis* and *V. phosphorescens*, cross absorption experiments were repeated several times. Modifying the technique, i.e., dilution of sera, incubation period, and temperature, did not alter the absorption results.

sorption of anti-*V. albensis* serum by *V. phosphorescens* resulted in an almost complete absorption of antibodies for both antigenic factors. In addition, *V. phosphorescens* almost completely absorbs from its homologous antiserum both *V. albensis* and *V. phosphorescens* agglutinins. These conclusions are reached from the agglutination reactions with the absorbed antisera (table 5).

Practically a complete absorption of anti-*V. albensis* serum occurs in the presence of *V. phosphorescens* antigen. A significant fundamental agglutinogenic similarity exists. Anti-*V. phosphorescens* serum will not only agglutinate both *V. phosphorescens* and *V. albensis* to full titer, but agglutinins of either species are almost completely absorbed by that organism.

TABLE 5

*The effect of absorbing cross-agglutinative immune sera with the heterologous antigens*

DILUTION OF ABSORBED SERUM		1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	1:20480	1:40960	CON- TROL
Antiserum-antigen	Agglutinating organism												
Anti-splendidum ab- sorbed by <i>B. sepiae</i>	<i>B. sepiae</i>	±	±	0	0	0	0	0	0	0	0	0	0
	<i>P. splendidum</i>	4	4	4	4	2	2	2	1	1	1	0	0
Anti-harveyi absorbed by <i>P. splendidum</i>	<i>P. splendidum</i>	1	±	0	0	0	0	0	0	0	0	0	0
	<i>A. harveyi</i>	4	4	4	4	3	1	1	1	0	0	0	0
Anti-albensis absorbed by <i>V. phosphorescens</i>	<i>V. phosphorescens</i>	1	1	1	0	0	0	0	0	0	0	0	0
	<i>V. albensis</i>	1	±	0	0	0	0	0	0	0	0	0	0
Anti-phosphorescens absorbed by <i>V. al- bensis</i>	<i>V. albensis</i>	1	1	0	0	0	0	0	0	0	0	0	0
	<i>V. phosphorescens</i>	2	2	2	1	1	1	0	0	0	0	0	0
Anti-phosphorescens absorbed by <i>V. phosphorescens</i>	<i>V. albensis</i>	1	1	±	0	0	0	0	0	0	0	0	0
	<i>V. phosphorescens</i>	1	0	0	0	0	0	0	0	0	0	0	0
Anti-albensis ab- sorbed by <i>V. albensis</i>	<i>V. albensis</i>	1	1	0	0	0	0	0	0	0	0	0	0
	<i>V. phosphorescens</i>	2	2	1	1	0	0	0	0	0	0	0	0

#### *Antigenicity of Filtrates After Cytolysis*

The antigenic properties of filtrates are of particular interest because the internal components of the cell rather than the entire cell are largely involved. Electron micrographs (Johnson, Zworykin, and Warren, 1943) have conclusively shown that distilled water cytolysis of luminous cells is rapid but is not accompanied by a complete disintegration of the cell wall. Furthermore, the end product of cytolysis is a cell structure which is for the most part devoid of internal components. The question arose as to whether the filtrates so prepared were capable of producing immune sera which would not only precipitate the filtrates themselves but would also agglutinate intact luminous cells.



Filtrates were prepared and standardized by the following procedure: 5 per cent by moist weight of luminous bacteria were cytolized in distilled water. The disappearance of luminescence was indicative of this cytolysis. The prepara-

TABLE 6

*Agglutination and cross agglutination of luminous bacteria with rabbit antisera prepared from cytolized filtrate antigens. Wherever single antigens are listed, no cross agglutination occurred*

ANTIGEN	1:2	1:4	1:8	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	CON- TROL
A. Anti-albensis														
<i>V. albensis</i> . . . . .	4	4	4	4	4	4	4	4	3	3	2	1	0	0
<i>V. phosphorescens</i>	4	4	4	4	4	4	4	4	4	2	2	1	0	0
B. Anti-phosphorescens														
<i>V. phosphorescens</i>	4	4	4	4	4	4	4	4	3	2	2	0	0	0
<i>V. albensis</i> . . . . .	4	4	4	4	4	4	4	4	2	2	2	±	0	0
C. Anti-harveyi														
<i>A. harveyi</i> . . . . .	4	4	4	4	2	1	1	0	0	0	0	0	0	0
<i>P. splendidum</i> . . . . .	2	2	1	0	0	0	0	0	0	0	0	0	0	0
D. Anti-splendidum														
<i>P. splendidum</i> . . . . .	4	4	4	3	2	2	2	1	0	0	0	0	0	0
<i>B. sepiae</i> . . . . .	3	2	1	0	0	0	0	0	0	0	0	0	0	0
E. Anti-sepiae														
<i>B. sepiae</i> . . . . .	4	4	4	4	3	1	1	1	0	0	0	0	0	0
F. Anti-fischeri														
<i>A. fischeri</i> . . . . .	4	4	4	3	3	2	1	±	0	0	0	0	0	0
G. Anti-phosphoreum														
<i>P. phosphoreum</i> . . . . .	2	2	2	1	1	1	0	0	0	0	0	0	0	0
H. Anti-pierantonii														
<i>B. pierantonii</i> . . . . .	4	4	4	3	3	2	1	1	0	0	0	0	0	0

tion of filtrates of the fresh-water forms were more difficult. However, *V. albensis*, the more resistant to cytolysis of the two, served as the distilled water "standard" for *V. phosphorescens*. In this way, some degree of standardization of the filtrate potencies is maintained. Clear filtrates were obtained with Berkefeld filters.

For the production of antisera, rabbits received intravenous injections of filtrates. Five successive injections were administered at 2-day intervals. Be-

ginning with 1 ml, the dosage was doubled with each injection. Following a rest period of 7 days, two 20-ml injections of filtrate were made at 3-day intervals. After a second rest period of 7 days, the animals were bled aseptically from the heart and the serum collected. Cross agglutination experiments were set up in the usual manner.

The results are summarized in table 6. With the exception of a general decrease in the agglutinating titers<sup>6</sup> and an antigenic difference in the *V. albensis*-*V. phosphorescens* cross agglutination, the data are in complete agreement with the straight agglutination reactions. A further interesting point was the almost identical cross agglutination results with either *V. albensis* or *V. phosphorescens*. This relationship, as shown in table 6, is discussed later.

### Precipitation Reactions

The antigen was prepared and standardized by the same method as employed in the antifiltrate agglutination study. Precipitin ring tests were performed with antisera prepared by injections of rabbits with both luminous cell antigen and filtrate antigen, respectively. For each test 0.2 ml of a 1:3 dilution of antiserum was carefully overlaid by 0.2 ml of antigen. Dilutions of antigen ranged from 1:1 to 1:160. Three controls detected any possibility of auto-precipitation.

The results of the cross precipitation reactions with luminous cell antibodies are found in table 7. It is apparent that the precipitin reactions reveal a mosaic of antigens within the bacterial cell in addition to a number of antigenic components which are common to several species.

Both *V. albensis* and *V. phosphorescens*, in addition to having mutual antigenic groups, still retain a similar titer relationship. Curiously enough, anti-*A. fischeri* serum has all the components necessary for the precipitation of any of the remaining species. In addition, a minor *A. fischeri* factor is present in the antisera of the other species. It is then valid to conclude that a common *A. fischeri* component is present in all species. The *A. harveyi*-*P. splendidum* cross precipitation is of a reciprocal nature. Furthermore, anti-*A. harveyi* serum will precipitate *P. phosphoreum*, *B. pierantonii*, and *B. sepiac* antigens. The *P. splendidum*-*B. sepiac* kinship is also of a reciprocal nature. It is to be noted that *B. pierantonii* serum possesses only one nonspecific component (*A. fischeri*).

The results of the cross precipitation tests with filtrate immune sera are shown in table 8. There is obviously much more cross precipitation than with the luminous cell immune sera. The *V. albensis*-*V. phosphorescens* reciprocal relationship is enhanced by the appearance of *A. harveyi* antibody in both sera. The antigenic makeup of *A. fischeri* and *B. sepiac* remain unchanged. Furthermore, anti-*A. harveyi* serum will duplicate the results of the anti-*A. fischeri* serum. In addition, *B. sepiac* and *P. splendidum* now possess a similar mosaic of antigenic components. The only marked change in antigenic structure is shown by *B. pierantonii*. With only one nonspecific precipitation factor when

<sup>6</sup> Although several additional injections of filtrate antigens were made, no appreciable increase in the agglutinating titers of the antisera resulted.





combined with luminous cell immune serum, the filtrate immune serum of this organism precipitates six additional filtrates. *P. phosphoreum* occupies an exactly similar kinship with both *B. sepiæ* and *P. splendidum*.

The precipitin tests appear to be highly sensitive in seeking out the component antigens in luminous bacteria filtrates. Prevalent nonspecific reactions in addition to weak precipitinogenic components are significant in the general antigenic system. It is evident from the enumeration of components present that the system is complex. Tests with luminous bacteria immune sera show a more specific antigen-antibody relationship than similar reactions with filtrate immune sera.

#### DISCUSSION

The results seem to indicate rather clearly that the six marine species of luminous bacteria studied, apart from minor agglutinins in two species (*A. harveyi* and *P. splendidum*), are fairly specific in their agglutinin reactions.

The fresh-water forms, *V. albensis* and *V. phosphorescens*, present a much closer cross agglutination relationship. They are also much more agglutinogenic than the marine forms. Immune sera will give a higher titer in a shorter immunization period. Whether this is due to the character of the antigenic reacting groups is not known.

With one exception, the results indicate that the specificity of the agglutinations with filtrate immune sera agrees with the results of luminous cell immune sera. This suggests that the agglutinin factors derived from luminous cells are all present in their filtrates and points to the classification of the latter as complete antigens.

It is apparent that the cultural as well as immunological characteristics of *V. albensis* and *V. phosphorescens* are for the most part similar. However, *V. phosphorescens* possesses a more intense luminescence, is more stable in suspension, and is more complete antigenically. These facts suggest the possibility that *V. albensis* is a "rough" variant of *V. phosphorescens*, although differences in colony form are not apparent.

The precipitation studies of luminous bacteria exhibit much less specificity than the agglutination reactions and, in addition, a marked nonspecific reaction at low titer. Nevertheless, the differentiation of the various species is facilitated by the higher titers of the homologous antisera.

Any consideration of the specific antigens involved in the precipitation reactions of luminous bacteria is more or less speculative. The marked group phenomena which characterize the results are of special interest since not merely different strains, or even species, but actually different genera are represented. This study is not comprehensive enough to justify any recommendations as to the most desirable place, in the classification of bacteria, for these organisms; nor for renaming or systematizing the group itself.

It is conceivable that these serological reactions are linked with similarity in the chemical structure of particular cellular components in which the luminescent system is of prime importance, since luminescence is perhaps the most outstand-

ing common property of the different organisms. A possible role played by the components of a common luminescent system of the organisms, in influencing antigenicity of the intact luminous cell, has not been established. Although no experiments were devised or conducted for the purpose of making a positive contribution to the problem of the mechanism of the luminescent system, comments on the subject can be presented on the basis of observations made during the course of this investigation.

Ballner (1907) reported no inhibition of luminescence with immune sera. Although Ninomya (1924) found an inhibition with immune sera, he showed that a correlation existed between the degree of agglutination and the inhibition of luminescence. It is highly probable, as suggested by Ninomya, that these inhibitions are due merely to an intense clumping of cells during agglutination reactions which interferes with an adequate gaseous exchange. The possibility of a specific antigen-antibody reaction which influences this inhibition was considered as a secondary factor by that author.

Harvey and Deitrich (1930) investigated the possible antigenic properties of the *Cypridina* luminescent system, i.e., luciferase and luciferin. They reported the production of an antiluciferase serum which combined with active luciferase. When this luciferase plus antibody was now added to luciferin, the luminescent reaction did not occur. It is apparent that the enzyme which is involved in the luminescent reaction of *Cypridina*, and which is assumed to be responsible for the same reaction in luminous bacteria, is antigenic.

The observation of numerous cases of both normal and immune sera in contact with luminous bacteria has revealed no evidence of a corresponding immunological interference with the light-emitting reactions in the living cells. In no instance was there an inhibition of luminescence, except by agglutination. The amount, and therefore potency, of such an enzyme antigen, of course, must also be considered in explaining an apparent absence of a specific luminescent antibody. Furthermore, luminescence is actually increased in the presence of both normal and immune sera. This is undoubtedly due to the addition of a nutrient substrate (Ninomya, 1924; Johnson, 1936; van Schouwenburg, 1938).

Thus, it is clear that an antiluciferase antibody is either not present in the immune serum or else, if it is present, it does not have access to the light-emitting enzyme in the intact bacterial cells. This point is of particular interest for its possible significance in connection with the problems of the site of oxidative systems in living bacteria. If it may be assumed that bacterial luciferase is antigenic, the results would indicate that the luminescent enzyme is not directly exposed to immunological combination at the cell surface.

#### SUMMARY

The antigenic properties of eight species of luminous bacteria are reported. Evidence was sought from the results of agglutination, agglutinin absorption, and precipitation.

Cross agglutination results indicate that specific agglutinogenic properties are present in *Achromobacter fischeri*, *Bacillus sepiac*, *Bacillus pierantonii*, and

*Photobacterium phosphoreum*. Cross reactions occur between *Achromobacter harveyi* and *Photobacterium splendidum*, between *Photobacterium splendidum* and *Bacillus septiae*, and between *Vibrio albensis* and *Vibrio phosphorescens*.

The nature of the *V. albensis*-*V. phosphorescens* kinship is different from the other cross reactions. At least two major antigens are present in *V. phosphorescens*, whereas *V. albensis* contains a major and minor antigen.

Agglutinin absorption tests confirm the existence of major and minor agglutinins in the case of *A. harveyi*-*P. splendidum*, and *P. splendidum*-*B. septiae*. Cross agglutinin absorptions with *V. albensis*-*V. phosphorescens* indicate a significant agglutinogenic similarity.

Filtrate antigens of luminous bacteria will give rise to agglutinins which exhibit specificities characteristic of luminous cell agglutinins.

A mosaic of common antigens is revealed by cross precipitation with both immune filtrate sera and immune luminous cell sera. An intensified group reaction is found with the immune filtrate sera. These results are discussed in relation to the chemical composition of cellular structures and a luminescence factor of possible antigenic significance.

The luminescence of living cells was not directly affected by any of the immunological reactions studied.

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# ANAEROBIC FERMENTATION OF CRYPTOSTEGIA LEAVES FOR RECOVERY OF RUBBER<sup>1</sup>

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*Cryptostegia grandiflora* R. Br. is a foliose tropical vine which bears a latex rich in rubber. The rubber can be obtained by cutting the long whiplike shoots or tapping the bark of the main trunk and collecting the latex which exudes. Recent work in this laboratory (Whittenberger, Brice, and Copley) has shown that the leaves of the plant also contain considerable quantities of rubber, which is localized in globules within the protoplasm of the chlorenchyma. This "cell rubber" comprises about 85 per cent of the total rubber of the leaves, the remaining 15 per cent being typical latex rubber, which is in the latex duct system of the leaf. The cell rubber cannot be recovered by tapping the latex system of the plant nor have satisfactory pebble-milling procedures been evolved analogous to those used for recovery of the rubber contained in the bark and wood of guayule. Solvent extraction is also unsatisfactory, as the yield of rubber is poor even after prolonged extraction unless the leaves are given an extensive chemical treatment prior to extraction.

Use of fermentation as an aid in the recovery of rubber from plants is not new. Lamb (1873) fermented milkweed prior to solvent extraction of the rubber, and Saunders (1875) found that a brief fermentation of *Asclepias cornuti* made the rubber more easily soluble. Aerobic fermentation of guayule prior to mechanical recovery of the rubber was patented by Spence (1933) and further investigated by Naghski, White, and Hoover (1944).

It therefore appeared probable that destruction of the cell walls by fermentation would make the rubber available for recovery. The conditions and procedure for this unique fermentation are discussed here. A report on aspects of the problem which bear on the recovery and technology of the rubber will be presented elsewhere (Hoover, Dietz, Naghski, and White).

## EXPLORATORY FERMENTATIONS

The fresh living leaves used in these preliminary experiments were from an F<sub>1</sub> hybrid of *Cryptostegia madagascariensis* × *C. grandiflora*.<sup>3</sup> The material

<sup>1</sup> Natural rubber from domestic sources. Paper no. 7.

<sup>2</sup> One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

<sup>3</sup> This material was obtained from the U. S. Plant Introduction Garden, Coconut Grove, Florida, through the courtesy of the Bureau of Plant Industry, Soils, and Agricultural Engineering, United States Department of Agriculture.

used in the remainder of the fermentation studies was dried leaves of *C. grandiflora*.<sup>4</sup>

Among the microorganisms tested were *Chaetomium globosum*, *Trichoderma lignorum*, *Aspergillus fumigatus*, *Penicillium* sp., *Aspergillus* spp., *Clostridium* sp.,<sup>5</sup> *Clostridium roseum*,<sup>5</sup> *Clostridium felsineum*,<sup>5</sup> *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Flavobacterium fecale*, and thermophilic cellulose fermenters. Several of these attacked the cell walls slowly, but two—*C. roseum* and *C. felsineum*—fermented the leaves readily, with considerable production of gas. Mild agitation disintegrated the fermented leaves and liberated a finely divided material which settled out as a green sediment. This sediment consisted of the coagulated contents of chlorenchyma cells devoid of cell walls, with the rubber globules embedded within them. Boiling with dilute alkali dissolved the protoplasts and liberated the rubber globules, which then rose to the surface. The rubber was also recovered readily from the protoplasts by solvent-extraction procedures. Photomicrographs of cross sections of a fresh living leaf, the isolated contents of the chlorenchyma cell, and the rubber-bearing globules recovered therefrom are shown in figure 1.

*Clostridium roseum* was selected for the investigation.

#### EFFECT OF VARIABLES ON THE FERMENTATION

**Assay method.** The effects of time, temperature, concentration of leaves, pretreatment of leaves, added nutrients, and condition of culture on the rate of retting were studied to determine optimum conditions for retting. The following method of assay was developed to evaluate the effects of these variables. Twenty-five grams (dry weight) of leaves in a 1.5-liter Erlenmeyer flask were given the desired pretreatment and made up to the desired volume with a mineral salt solution (Allison and Hoover, 1934) or water, and then inoculated with a 10 per cent volume of an 18-hour broth culture. The flask was equipped with a water trap to permit flushing out with nitrogen and also to allow the escape of fermentation gases. During incubation, it was shaken occasionally to keep "heading" at a minimum. Except for the temperature studies, all assays were incubated at 39 to 40 C. At the end of incubation, the contents of the flask were transferred to a 1-quart fruit jar and shaken for 30 minutes in a mechanical shaker. The disintegrated leaves were screened on a 20-mesh sieve. The residue was diluted with 200 ml of water and screened again. The residue on the screen was dried at 100 C and weighed. The relative efficiency of the fermentation, expressed as "degree of retting," was calculated as follows:

$$\text{Degree of retting} = \frac{\text{g of residue from standard treatment}}{\text{g of residue from experimental treatment}}$$

The weight of residue from leaves extracted for three 5-minute periods with boiling water, made up to 5 per cent concentration, inoculated, and incubated

<sup>4</sup> This material was obtained through the courtesy of the Atkins Institution of the Arnold Arboretum of Harvard University, Soledad, Cuba.

<sup>5</sup> Cultures of *C. roseum* A 42, *C. felsineum* A 41, and *C. sp.* A 40 were obtained through the courtesy of Dr. E. McCoy of the University of Wisconsin.

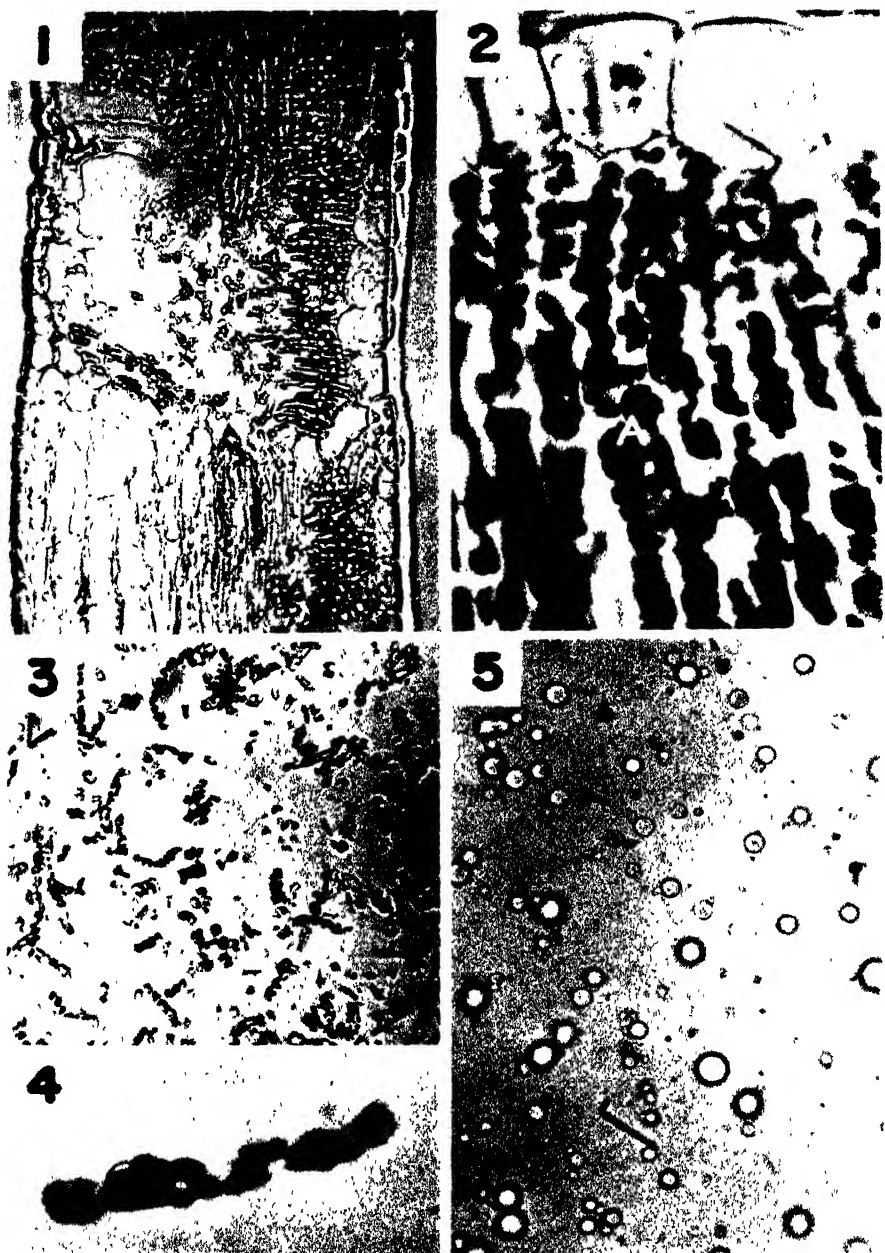


FIG. 1. PHOTOGRAPHS OF MATERIAL PREPARED FROM AN  $F_1$  HYBRID OF  
*C. MADAGASCARIENSIS*  $\times$  *C. GRANDIFLORA*

1. Cross section of preserved specimen of mature leaf; unstained. 130  $\times$ . Rubber globules visible in the chlorenchyma.
2. Cross section of fresh, living, senescent leaf. 460  $\times$ . (A) Rubber-bearing globules and chloroplasts within the palisade cells. (B) Upper epidermis.
3. Protoplasts from retted leaves. 130  $\times$ .
4. A protoplast from retted leaf. 1000  $\times$ .
5. Suspension of rubber-bearing globules isolated from protoplasts by alkaline digestion of the protoplasts. 460  $\times$ . Many of the globules are not in focus.

at 39 to 40 C for 2 days was taken as the standard. This weight depended upon the particular batch of leaves fermented; consequently controls were run as part of each experiment. Leaching out soluble constituents during pretreatment and incubation of the uninoculated control produced a loss in weight equivalent

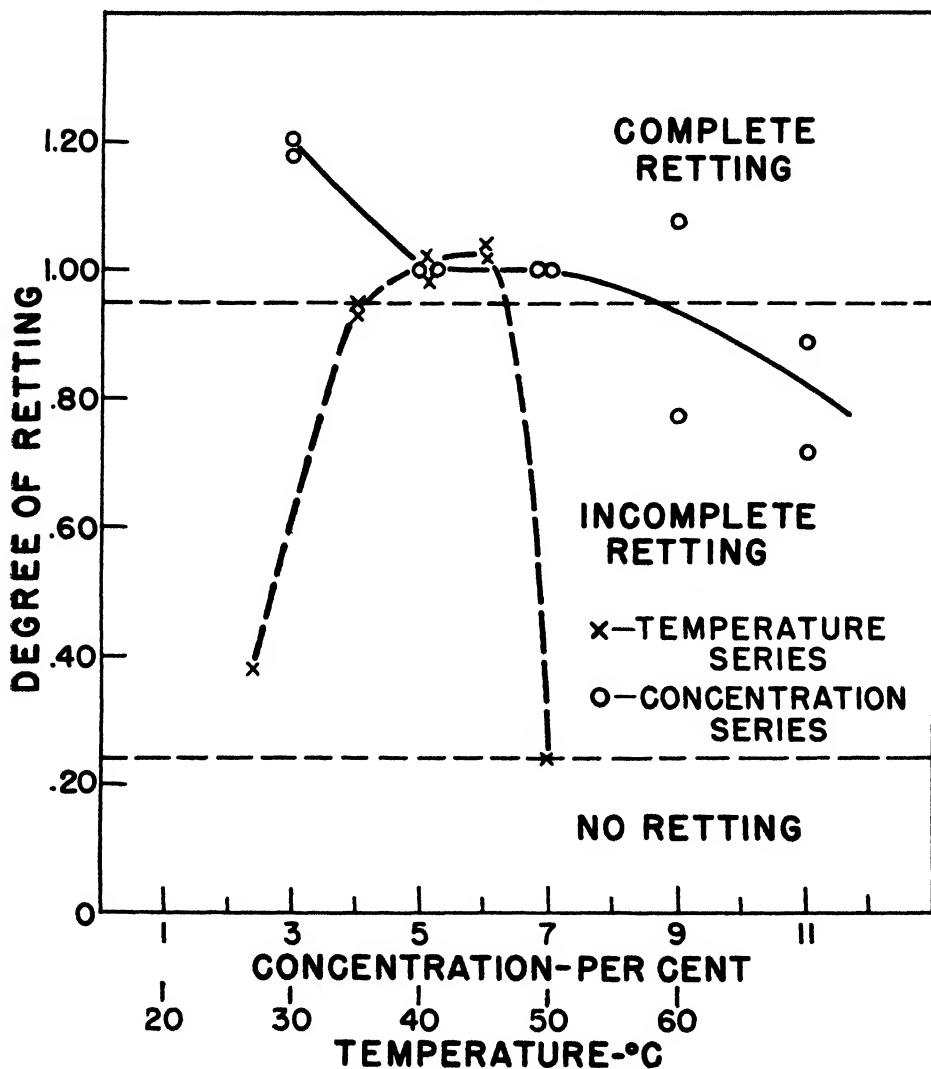


FIG. 2. EFFECT OF TEMPERATURE AND CONCENTRATION ON EXTENT OF RETTING OF *CRYPTOSTEGIA* LEAVES BY *CLOSTRIDIUM ROSEUM* IN TWO DAYS AT 39 TO 40 C

to a degree of retting of 0.24; therefore values below this point represent no retting. Microscopic examination of samples giving a value of 0.95 or above showed that retting was essentially complete. This assay method enabled us to evaluate the effect of a large number of factors upon the fermentation and quickly determine optimum conditions. The excellent reproducibility of results is shown later in figure 2.

*Production of inoculum.* A satisfactory medium for culturing the inoculum consisted of 16 ml of blackstrap molasses, 1 g of glucose, 5 ml of corn steep liquor, and 1 g  $(\text{NH}_4)_2\text{SO}_4$  per liter of mineral salts solution<sup>6</sup> (Allison and Hoover, 1934); the reaction was adjusted to pH 6.8 to 7.2 with 10 per cent sodium hydroxide. No attempt was made to simplify this medium further. Growth was rapid; 12- to 18-hour cultures gave satisfactory inocula for fermentations. Inocula more than 24 hours old became sluggish and showed a prolonged lag period. Microscopic examination of the latter showed spores to be predominant. The cultural and biochemical characteristics of *C. roseum* have been reported by McCoy and McClung (1935).

*Subculturing.* During exploratory work on the decomposition of *Cryptostegia* leaves, the culture was maintained in an actively growing state by transferring it to a fresh tube of corn mash every other day rather than starting a fresh culture from the dry-soil spore suspension. After a month of such treatment the culture

TABLE 1  
Effect of subculturing *C. roseum* on rate of retting of *Cryptostegia* leaves

AGE OF CULTURE FROM STOCK*	NUMBER OF CONSECUTIVE SUBCULTURES IN CORN MASH	DEGREE OF RETTING AFTER	
		2 days	3 days
<i>days</i>			
2	1	1.00	1.10
44	3	0.90	1.04
44	5	0.62	0.76
13	7	0.59	0.76
44	22	0.58	0.58

\* Stock culture was a spore suspension in dry sterile soil.

fermented the leaves poorly. Table 1 gives data from an experiment designed to show the effect of frequency of transferring *C. roseum* in corn mash on the loss of retting ability. A culture freshly initiated from the dry-soil spore suspension produced complete retting in 2 days. Cultures passing through more than the initial transfer gave progressively lower retting values. Cultures permitted to go into the spore state did not continue to lose retting ability, as shown by the 44-day cultures that sporulated in the corn mash after the third and fifth transfer.

However, a stock culture maintained in the spore state in dry sterile soil retained its activity and viability for more than 18 months. McCoy *et al.* (1926) reported that cultures developed from soil preparations were more active than those from corn mash.

*Pretreatment of the leaves.* The effects of various leaching procedures and of the medium are presented in table 2. Excellent results were obtained when the leaves were given three 5-minute extractions or one 15-minute extraction with

<sup>6</sup> The solution contained 0.3 g  $\text{KH}_2\text{PO}_4$ , 0.7 g  $\text{K}_2\text{HPO}_4$ , 0.2 g  $\text{NaCl}$ , 0.2 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.1 g  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  per liter.

boiling water. Removal of extractives increased the rate of retting, presumably owing to the removal of inhibiting materials. It was also found that the leached leaves were fermented equally well whether mineral salts solution or tap water was used as the medium. Apparently sufficient minerals and nutrilities for optimum growth were carried over in the inoculum. Therefore, tap water was used in all large-scale experiments.

*Concentration of solids.* The maximum concentration of leaves which could be fermented in 2 days was determined. The results are shown in figure 2. The reproducibility of data obtained at 3, 5, and 7 per cent concentrations (based on original unleached weight of dry leaves) is illustrated by the superposition of

TABLE 2

*Effect of pretreatment and medium on degree of retting of Cryptostegia leaves by C. roseum*

CONCENTRATION OF LEAVES	PRETREATMENT	MEDIUM	DEGREE OF RETTING
%			
3	1 60-min steep at 62 C, not drained	tap water	0.66
3	1 60-min steep at 62 C, drained	mineral salts	0.74
3	1 60-min steep at 62 C, drained	tap water	0.76
5	3 20-min steeps at 55 C, drained	mineral salts	0.74
5	1 1-min boil, drained	mineral salts	0.84
5	1 5-min boil, drained	mineral salts	0.90
5	1 10-min boil, drained	mineral salts	0.95
5	1 15-min boil, drained	mineral salts	1.00
5	1 15-min boil, not drained	tap water	0.77
5	2 5-min boils, drained	mineral salts	0.96
5	3 5-min boils, drained (standard treatment)	mineral salts	(1.00)
3	3 5-min boils, drained	mineral salts	1.00
7	3 5-min boils, drained	mineral salts	1.00
3	4 5-min boils, drained	mineral salts	1.05
3	4 5-min boils, drained	tap water	1.00

points for different experiments. Complete fermentation of the 7 per cent slurry was obtained. The inconsistent results at 9 and 11 per cent concentrations were due to the difficulty encountered in keeping the leaves submerged. Unless the leaves were submerged they fermented slowly. In large-scale experiments concentrations above 5 per cent were difficult to handle with the equipment available. Undoubtedly the fermentation could be carried out with a higher solids content in equipment designed for the purpose.

*Temperature.* Temperature had a marked effect on the fermentation (figure 2). Retting was substantially complete in 2 days at 35 to 45 C. At 28 C the decomposition proceeded slowly; and at 50 C no retting occurred. McCoy and McClung (1935) found that the range of temperature at which this organism

grew was from 8 to 62 C. They also found that the fermentation of corn mash at the higher temperatures investigated (54 to 62 C) was incomplete, and, of course, at low temperatures growth was slow.

**Contaminants.** *C. roseum* established an essentially pure culture when incubated anaerobically, even with unsterilized leaves. It is therefore not especially sensitive to bacterial competition. This is in agreement with the results obtained by Dr. E. McCoy in retting plants for bast fibers with this organism (personal communication).

Action antagonistic to *C. roseum* was observed when attempts were made to ret a sample of molded leaves. Growth of bacteria was light, and the cell walls of the leaves were not decomposed.

#### FERMENTATION PROCEDURE

On the basis of the foregoing results, large-scale fermentations were carried out in 40- and 120-gallon volumes. Many runs were made to prepare sufficient material for chemical and physical tests. A typical run will be described in which the leaves were retted in a 50-gallon barrel under nearly optimum conditions. A 7.45-kg lot of dry leaves was extracted with boiling water, made up to 140 liters with tap water (40 C), and inoculated by introducing 10 liters of an 18-hour culture of *C. roseum* at the bottom. The barrel was then covered with a gasketed lid and kept anaerobic by leading in carbon dioxide gas generated from solid carbon dioxide in a Dewar flask. Incubation was carried out at 35 to 37 C for 2 days. Difficulty with "heading" was overcome by using a motor-driven, low-pitch propeller blade, which just swept the surface of the liquor and revolved at 6 rpm. This intermittently submerged the leaves that were pushed out of the liquid by the gases and also produced a gentle rocking action, which dislodged the gas and permitted it to escape.

After two days' incubation, the leaves were well digested and disintegrated. The slurry was passed over a vibrating screen (80 by 80 meshes to the inch). The liquid and the protoplasts passed through, but the bagasse (cuticle, veins, and small stems) remained on top. Because the latex ducts in *Cryptostegia* leaves are long and closely associated with the veins, they were trapped in the bagasse. The bagasse was dispersed in water to half the original volume and again screened to recover the protoplasts that were trapped mechanically. The bagasse was freed of excess water by pressing and then dried. The protoplasts (sp gr 1.17 to 1.27) were recovered from the liquor in which they were suspended by gravity settling and decantation; a slurry containing about 4 to 7 per cent solids was obtained. The slurry was further freed of soluble materials by diluting with water, settling, and decanting.

The data presented in table 3 show that the protoplasts amounted to one fourth of the original weight of leaves, and contained more than three fourths of the rubber, whereas the bagasse fraction amounted to less than one eighth of the original weight and contained less than one fourth of the rubber. Microscopic examination showed that the protoplasts were essentially free of latex ducts. Since the bagasse fraction included a small portion of incompletely dis-



integrated leaves and also mechanically held some released protoplasts, it contained an appreciable quantity of cell rubber. These results are in agreement with those of Whittenberger, Brice, and Copley, who found about 10 to 15 per cent of the total rubber of the leaves to be in the latex-duct rubber fraction. The loss in dry weight effected by the fermentation increased the over-all concentration of rubber from 4.2 to 11.2 per cent. The rubber was determined by a method developed at this laboratory (unpublished).

TABLE 3

*Distribution of rubber in protoplasts and bagasse of Cryptostegia leaves fermented by C. roseum*

	ORIGINAL LEAVES	PROTOPLASTS	BAGASSE	RETTING LOSS
Dry weight, kg. . . . .	7.45	1.86	0.87	4.72
% of original leaves . . . . .		25.0	11.7	63.3
Rubber content, %* . . . . .	4.2	12.7	8.1	
% of original rubber in— . . . . .		76.0	22.6	1.4

\* Moisture-free basis

#### DIGESTION OF PROTOPLASTS

In addition to the rubber and resin the dried protoplasts contained 5.5 to 7.3 per cent nitrogen (depending on the composition of original leaves), equivalent to 34 to 45 per cent protein. Considering that the protoplasts are proteinaceous, it would appear that they should be decomposed by microorganisms or proteolytic enzymes. They proved, however, to be refractory. Attempts to digest the protoplasts with papain were not successful. When a dilute suspension of protoplasts was first adjusted with NaOH so that after boiling or autoclaving at 15 pounds for 20 minutes the pH was between 8.0 and 8.5, the rubber globules were liberated by fermentation with *P. aeruginosa*, *F. fecale*, and *B. subtilis*. Adjusting the reaction with alkali did not liberate the rubber globules but changed the structure of the protoplasts so that these bacteria were able to digest them. Hydrochloric, sulfuric, and oxalic acids in concentrations up to 10 per cent did not digest the protoplasts (10 per cent suspension), even after 5 hours' boiling, and a 40 per cent solution of urea also did not dissolve them to any appreciable extent. Boiling with dilute aqueous alkali (NaOH or KOH) dissolved the protoplasts, however, and released the rubber globules. The concentrations of dilute aqueous alkali and of protoplasts which permitted dissolution of the protoplasts are shown in table 4.

#### FERMENTATION OF CELLULOSE BY CLOSTRIDIUM ROSEUM

Microscopic examination of retted leaves showed that the parenchyma cell walls were digested and the protoplasts liberated, indicating that *C. roseum* was capable of fermenting cellulose. McCoy and McClung (1935) found that *C. roseum* did not ferment filter paper cellulose in tryptone broth. We have confirmed this result. However, concerning growth on potato slants, they state:

"The disintegration of tissue here differs from reaction of *C. acetobutylicum* and suggests fermentation of pectin or cellulose or both." Therefore, to correlate microscopic observations with chemical analysis, samples of leaves were analyzed before and after fermentation for 4 days. The residue after fermentation was washed thoroughly to remove soluble substances, and the total insoluble fraction was recovered quantitatively. For comparison another *Clostridium* culture (A 40) which does not produce satisfactory retting was used in a similar experiment. Determinations of crude hemicellulose and crude cellulose were

TABLE 4

*Effect of concentration of alkali and protoplasts on rate of solution of protoplasts*

PROTOPLAST CONCENTRATION	CONCENTRATION OF SODIUM HYDROXIDE IN LIQUOR	TIME OF BOILING*	RATIO OF PROTOPLASTS TO SODIUM HYDROXIDE
%	%	min.	
1.6	0.3	10	5.3
5.0	0.5	15	10
5.0	1.0	5	5
10.0	1.0	20	10
10.0	1.5	15	6.6

\* Minimum time necessary to effect complete dissolution of protoplasts as observed microscopically.

TABLE 5

*Composition of leaves before and after retting for four days with C. roseum and with Clostridium A 40*

CONSTITUENT	BEFORE RETTING	AFTER RETTING BY C. ROSEUM		AFTER RETTING BY CLOSTRIDIUM A 40	
		%	% LOSS	%	% LOSS
	%				
Dry weight .....	100	38.5	61.5	53.0	47.0
Ash .....	16.96	4.84	89.1	5.59	82.6
Nitrogen .....	2.67	5.35	22.9	4.46	11.2
Crude hemicellulose .....	2.65	2.70	61.1	2.53	49.5
Crude cellulose .....	9.25	6.03	75.0	9.96	43.1

made before and after fermentation. Results of the analyses are presented in table 5. Ash and nitrogen were determined by the methods of analysis of the Association of Official Agricultural Chemists for plant materials (1940). The crude hemicellulose was determined by a modification of the methods of Potts and Bridge (1937); the crude cellulose was determined by a modification of the method used by Vladesco (1940). Before these methods could be applied, the plant had to be freed of rubber and pectin by digestion with ammonium oxalate followed by successive extractions with toluene, alcohol, and benzene.

Although the fermentation with both organisms proceeded rapidly, with vigorous evolution of gas, the analytical results, together with the differences in

the effectiveness of release of the protoplasts, allow certain distinctions to be made. *C. roseum* produced a definitely greater loss in weight, nitrogen, and crude cellulose than did *Clostridium* A 40. Both organisms caused about the same loss in crude hemicellulose. The protoplasts were not released by fermentation with culture A 40, even when the leaves were agitated mechanically. It appears therefore that the digestion of hemicellulose and the more readily available cellulosic materials does not permit liberation of the protoplasts from the cells. Apparently *C. roseum* attacks the structural cellulose of the parenchyma cell wall, thus releasing the coagulated cell contents. However, it does not decompose the cell walls of the veins and midribs, thus leaving a "skeleton" of the vascular system.

#### DISCUSSION

The voluminous literature dealing with biochemical decomposition of cellulose, hemicelluloses, etc., has been reviewed by Thaysen and Bunker (1927), by Norman (1937), and by Waksman (1932, 1940). Buswell and Hatfield (1939) have discussed the use of anaerobic fermentation for the production of combustible gas from cellulose and related constituents of agricultural residues. To our knowledge, fermentation has not been used for the segregation of the chlorenchyma cell contents.

The digestion of the cell wall, together with the chemical data obtained, strongly suggests that the cellulosic materials and related constituents of the cell wall were attacked. Moreover, it is well known that the retting of plants for bast fibers by related organisms has to be closely controlled to prevent over-retting and consequent loss of strength of the fiber. It is apparent that after the pectinaceous substances have been decomposed, the organism gains access to the cellulosic fibers, thereby weakening them by partial digestion. The fact that the retting organisms have not been considered as having cellulose-digesting ability further indicates that action on filter paper may be too exclusive a criterion. Fuller and Norman (1943) have shown that cornstalk cellulose, especially when it contains xylans, is more readily utilized than filter paper and is vigorously decomposed by organisms that utilize filter paper slowly. McClung (1943) has also shown that the chromogenic anaerobes, especially of the *C. felsineum* type, are prevalent in nature and can be readily demonstrated by proper techniques. Our data further emphasize the possibility that this group of organisms plays a more important role in the decomposition of plant materials in nature than has been hitherto suspected.

Application of this unique fermentation in the study of other problems is at once apparent. It has already been utilized by Whittenberger, Brice, and Copley to prepare leaf skeletons in their study of the distribution of rubber in *Cryptostegia* leaves; and similar skeletons of other leaves have been prepared. In the separation and chemical study of the protoplasts from leaves of various plants now under investigation, the techniques used here have been applied without modification.

## SUMMARY

Anaerobic decomposition of *Cryptostegia* leaves for the recovery of rubber was studied. A two-day fermentation of preboiled *Cryptostegia* leaves by *Clostridium roseum* at 35 to 45 C effected a loss of 60 per cent in the original dry weight of leaves and a 75 per cent loss in the crude cellulose. Consequently, the rubber content of the product was more than two and one-half times that of the original material, on a moisture-free basis. Parenchyma cell protoplasts were liberated by destruction of their cell walls. Screening fermented leaves sufficed to separate the protoplasts containing the cell rubber from the latex rubber in the other fraction comprising veins, epidermis, and cuticle.

The protoplasts were resistant to action by acids, but were dissolved by dilute alkali; the liberated rubber globules rose to form a cream.

It has been established microscopically and chemically that *C. roseum* ferments the cellulosic fractions of leaves *in situ*.

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# A SERIES OF NEW SULFONAMIDES WHICH ARE UNAFFECTED BY *p*-AMINOBENZOIC ACID

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Probably the greatest contribution to the field of chemotherapy was Domagk's introduction of prontosil (chrysoidin) in 1935. Trefouel *et al.* (1935) soon theorized that in the tissues prontosil was broken down at the azo linkage to triaminobenzene and *p*-aminobenzenesulfonamide, and that this latter group was the active part of the prontosil molecule. The introduction of prontosil and its active principle, sulfanilamide, opened the way for successful treatment of streptococcal, gonococcal, and meningococcal infections. Inasmuch as pneumococcus infections did not respond to sulfanilamide therapy, it remained for Whitby (1938) to introduce sulfapyridine, a sulfonamide effective in the treatment of pneumococcus infections as well as those previously mentioned. With this impetus given to sulfonamide research, several new and important compounds were soon introduced: sulfathiazole by Fosbinder and Walter (1939), sulfadiazine by Roblin *et al.* (1940), and sulfaguanidine by Marshall *et al.* (1940). Up to this period none of the sulfonamide compounds were found to have any appreciable effect against certain other groups of organisms, notably the anaerobic clostridia commonly associated with war-wound infections.

Miller *et al.* (1940) synthesized *p*-aminomethylbenzenesulfonamide (sulfamylon<sup>1</sup>) which was subsequently found by Klarer (1941) to be highly effective against certain anaerobic bacteria. Moreover, Schreus (1942) found that *p*-aminomethylbenzenesulfonamide was unaffected by *p*-aminobenzoic acid, an attribute not associated with the common sulfonamides described heretofore. These observations led one of us (Lawrence, 1944, 1945) to conduct an extensive study on the *in vitro* activities of *p*-aminoalkylbenzenesulfonamides against a variety of microorganisms. It also stimulated an interest in the search for other possible sulfonamides which are unaffected by *p*-aminobenzoic acid. Compounds of this type were found when a group of sulfanilylanilides was investigated. The main representative of this group is sulfanilyl-3,5-dibromoanilide. A discussion of *p*-aminobenzoic acid relationships to the latter will be presented in another section of this report.

To our knowledge, there is no reference in the literature to the antibacterial effects of di-halogen-substituted sulfanilylanilides. Suter and Weston (1940), however, described the preparation of a mono-halogenated sulfanilylanilide, sulfanilyl-4-fluoroanilide, and noted that it showed a slight effect against streptococcal infections in mice. Marchant *et al.* (1942) described a series of 38 N'-substituted sulfanilamides, among which was sulfanilyl-4-bromoanilide.

<sup>1</sup> Trade name of Winthrop Chemical Company, Inc. It is known abroad as "marfanil" or "mesudin" and in the United States also as "homosulfanilamide."

They found that this compound did not possess antibacterial activity against any of their test organisms. In both the foregoing instances only a single halogen atom is attached to the anilide portion of the molecule. Long and Burger (1941) synthesized a series of iodinated aromatic compounds and included in their group sulfanilyl-2,4-diiodoanilide. However, their studies did not include data on antibacterial activity.

When preliminary tests showed that the antibacterial activity of sulfanilyl-3,5-dibromoanilide was unaffected by *p*-aminobenzoic acid, studies were made to determine the *in vitro* effects of the compound upon a wide variety of bacteria.

TABLE 1  
*Chemical names and molecular weights of compounds*

COMPOUND NO.	CHEMICAL NAME	MOL. WT.
1	Sulfanilyl-3,5-dibromoanilide*	406.1
2	Sulfanilyl-3,5-dichloroanilide*	317.2
3	Benzenesulfon-3,5-dibromoanilide*	391.1
4	Aminomethylbenzenesulfon-3,5-dibromoanilide†	420.1
5	Ethanesulfon-3,5-dibromoanilide†	343.1
6	3,5-dibromoaniline*	250.9
7	3,5-dibromoaniline hydrochloride	287.4
8	Sodium salt of sulfanilyl-3,5-dibromoanilide	428.1
9	Sodium salt of sulfanilyl-3,5-dichloroanilide	339.2
10	Sodium salt of benzenesulfon-3,5-dibromoanilide	413.1
11	Sodium salt of benzenesulfon-2-chloroanilide	289.7
12	Sodium salt of benzenesulfon-3-chloroanilide	289.7
13	Sodium salt of benzenesulfon-4-chloroanilide	289.7
14	Sodium salt of benzenesulfon-3,4-dichloroanilide	324.2
15	Sodium salt of benzenesulfon-2,5-dichloroanilide	324.2
16	Sodium salt of benzenesulfon-3,5-dichloroanilide	324.2
17	Sodium salt of N <sup>4</sup> -acetylsulfanilyl-3,5-dichloroanilide	381.2
18	Sodium salt of N <sup>4</sup> -acetylsulfanilyl-3,5-dibromoanilide	470.1

\* = Compound first dissolved in a few ml of alcohol preparatory to initial broth dilution.

† = Compound first dissolved in a few ml of N/1 Na<sub>2</sub>CO<sub>3</sub> preparatory to initial broth dilution.

All other compounds dissolved directly in broth.

Assuming that the activity of the compound was not due to the NH<sub>2</sub> group in the *para*-position, but rather to the other components of the molecule, an analogous compound was prepared and tested in which the amino group was lacking, i.e., benzenesulfon-3,5-dibromoanilide.<sup>2</sup> Another compound was studied in which the bromine atoms were replaced by two chlorine atoms, or sulfanilyl-3,5-dichloroanilide. Due to the relative insolubility of the compounds mentioned, their sodium salts were prepared and were compared with the parent anilides for activity. In addition, derivatives were tested in which the two halogen

<sup>2</sup> We wish to express our appreciation to Dr. H. Kaplan and Mr. G. Leubner for the preparation of all the anilides mentioned in this report. An account of their methods of synthesizing the compounds will be published elsewhere.

atoms were in various positions on the ring. Several mono-halogenated compounds, two acetyl derivatives, and the simple 3,5-dibromoaniline were added to the series. Ethanesulfon-3,5-dibromoanilide and the *p*-aminomethylbenzenesulfonamide analogue of the "type-compound," aminomethylbenzenesulfon-3,5-dibromoanilide, completed the group. A list of the compounds together with their molecular weights is given in table 1. In figure 1 are given

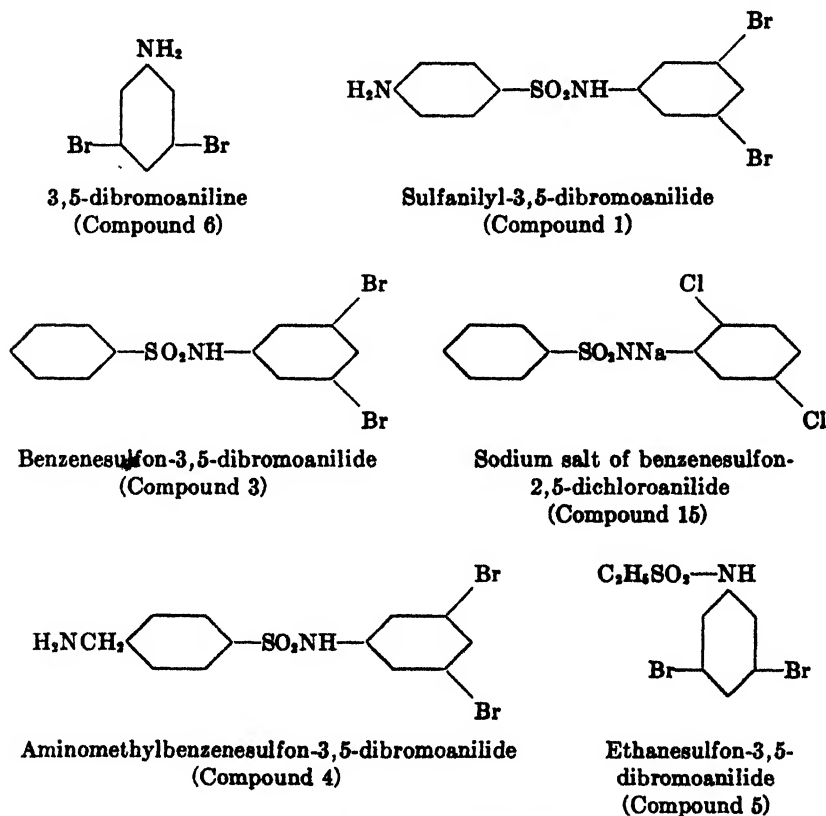


FIG. 1. STRUCTURAL FORMULAE OF REPRESENTATIVE COMPOUNDS

the structural formulae of several representative types of the benzenesulfonic acid derivatives.

#### METHODS

With the exception of the studies in which the gonococcus was used, the general method of testing the compounds was as follows: An initial dilution of 1:1,000 of each compound was prepared in a broth medium. Compounds which were relatively insoluble in water were first dissolved in a few milliliters of alcohol or, in some instances, in a molar solution of sodium carbonate preparatory to making the initial broth dilution (table 1). From the latter, serial dilutions were prepared in broth up to and including 1:512,000. The tubes were auto-



claved at 10 pounds for 10 minutes. In the pneumococcus, meningococcus, and streptococcus tests normal horse serum was added to the medium to give a final concentration of 0.1 per cent. The inoculum added to each tube was one 4-mm loopful of a 24-hour broth culture of the test organism. The organisms used and the media in which they were tested are as follows:

Pneumococcus type I	}	.... Veal infusion glucose broth + 0.1% horse-serum
Pneumococcus type II		
Pneumococcus type III		
<i>Streptococcus pyogenes</i> (C-203)		
<i>Staphylococcus aureus</i>		
<i>Eberthella typhosa</i>	}	..... Beef extract broth
<i>Salmonella paratyphi</i>		
<i>Shigella paradysenteriae</i> (Hiss)		
<i>Vibrio cholerae</i>		
<i>Clostridium welchii</i>	}	..... Brewer's fluid thioglycollate medium
<i>Clostridium tetani</i>		
Meningococcus group I	}	Brain heart infusion broth (Difco) + 0.1% horse serum
Meningococcus group IIa		

After inoculation all tubes were incubated at 37 C and examined for visible growth at intervals of 24, 48, and 72 hours. Lack of growth after 24 hours' incubation was considered evidence of bacteriostasis. Tubes which failed to show growth at the end of 72 hours were tested for bactericidal activity by transferring 3 loopfuls of the drug organism broth mixture to a broth medium lacking the drug. Failure of growth to appear in the subculture tube was taken as evidence of bactericidal action on the part of the drug in the original medication tube. The results of tests against the gram-positive cocci, the gram-negative and gram-positive bacilli, and the gram-negative cocci are given in tables 2, 3, and 4, respectively.

The test procedure was modified somewhat in the studies on gonococci. Dilutions up to and including 1:150,000 of the compounds were prepared in a medium of the following composition:

Proteose-peptone, Difco.....	15 g
Glucose.....	2 g
Soluble starch ..	10 g
NaCl.....	5 g
Na <sub>2</sub> HPO <sub>4</sub> .....	3 g
Gelatin .....	20 g
Distilled water.....	1000 ml

After autoclaving, each tube was inoculated with a loopful of a saline suspension of gonococcal cells. The suspension was prepared by washing the growth from a 24-hour glucose starch agar slant (Difco) with 5 ml of physiological saline solution. The inoculated tubes were incubated at a temperature of 37 C. Subcultures were made from these tubes to fresh glucose starch agar after the organisms had been exposed for 24 and 48 hours to the test compounds. The subculture tubes were incubated at 37 C for 72 hours, and the amount of growth

recorded. Complete absence of growth on the slants was taken as evidence of a bactericidal effect of the drug upon the organisms in the original mixture. The results of the tests against gonococci are part of table 4.

## RESULTS

As indicated in table 2, the gram-positive cocci are affected to the greatest extent by sulfanilyl-3,5-dibromoanilide, sulfanilyl-3,5-dichloroanilide, and their sodium salts (compounds 1, 2, 8, and 9). The removal of the amino group (compounds 3 and 10) results in a slight loss of activity. It would appear, therefore, that sulfanilylanilides or benzenesulfonanilides having two halogen

TABLE 2  
*Highest dilution of compound showing antibacterial activity*

COMPOUND NO.	PNEUMOCOCCUS						STREPTOCOCCUS PYOGENES C203		STAPHYLOCOCCUS AUREUS	
	Type I		Type II		Type III		Bs	Bc	Bs	Bc
	Bs	Bc	Bs	Bc	Bs	Bc				
1 & 8	128	64	128	128	128	64	32	32	64	<1
2 & 9	128	64	256	128	128	64	32	32	32	<1
3 & 10	64	16	128	32	64	16	8	<1	64	<1
4	8	4	8	8	8	4	8	2	4	<1
5	4	2	4	4	8	4	4	2	1	<1
6 & 7	4	4	4	4	4	2	4	4	8	4
11	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
12	8	8	16	8	8	4	4	4	4	2
13	4	2	8	4	8	4	4	4	4	4
14	16	16	32	16	<1	<1	8	<1	4	<1
15	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
16	16	8	16	8	16	8	8	8	<1	<1
17	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
18	16	16	32	16	<1	<1	<1	<1	<1	<1

Figures represent dilutions bacteriostatic (Bs) or bactericidal (Bc) as expressed in thousands, i.e., 128 = 1:128,000.

<1 = Concentrations greater than 1:1,000 not tested.

atoms in the 3,5 position are considerably more effective for this group of organisms than those with the halogens in the other positions on the anilide portion of the molecule.

With the anaerobic clostridia (table 3), it may be noted that the compounds in which the bromine or chlorine atoms are in the 3 or 3,4 or 3,5 positions on the anilide portions of the molecules exhibit the greatest antibacterial effect (compounds 1, 2, 3, 8, 9, 10, 12, 14, and 16). With but few exceptions, the compounds have little, if any, effect against the gram-negative enteric bacilli. The only consistent results which may be considered significant are obtained with aminomethylbenzenesulfon-3,5-dibromoanilide, ethanesulfon-3,5-dibromoanilide, and the simple 3,5-dibromoanilines (compounds 4, 5, 6, and 7).

In general the anilides are effective against the meningococci (table 4). Most

of them are superior to sulfanilamide and *p*-aminomethylbenzene sulfonamide but less active than sulfathiazole. It should be noted that sulfanilyl-3,5-dibromoanilide (compound 1) compares in activity with sulfathiazole.

Throughout all these tests the single halogen compound, benzenesulfon-2-chloroanilide (compound 11) and the N<sup>4</sup> acetyl derivatives of either sulfanilyl-3,5-dichloro- or sulfanilyl-3,5-dibromoanilide (compounds 17 and 18) show practically no antibacterial activity, regardless of the organisms used. Aside from these three compounds, the remainder have a definite inhibitory action against one organism or another. There is, furthermore, a suggestion of specificity of one type of compound for a certain group of organisms.

TABLE 3  
*Highest dilution of compound showing antibacterial activity*

COMPOUND NO.	CLOSTRIDIUM				ESBERTHELLA TYPHOSA*		SHIGELLA PARADYSENTERIAE		VIBRIO CHOLERAE	
	welchii		tetani		Bs	Bc	Bs	Bc	Bs	Bc
	Bs	Bc	Bs	Bc						
1 & 8	32	16	32	16	<1	<1	16	<1	32	<1
2 & 9	8	8	16	16	<1	<1	16	<1	32	4
3 & 10	32	32	32	32	<1	<1	<1	<1	<1	<1
4	2	1	2	2	4	1	16	2	16	8
5	4	<1	4	4	4	<1	8	2	16	2
6 & 7	2	2	4	4	8	8	16	8	16	16
11	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
12	8	4	8	8	<1	<1	1	1	8	8
13	8	8	8	8	<1	<1	1	<1	8	8
14	16	16	32	32	<1	<1	<1	<1	4	<1
15	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
16	16	16	16	16	<1	<1	<1	<1	4	<1
17	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
18	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

See legend under table 2.

\* Identical results were obtained with *Salmonella paratyphi*.

Of particular interest are the results obtained with the various strains of gonococci. Whereas strains Led, 91, and 66 are relatively sensitive to the sulfonamides, strain Spr appears to be of the "sulfonamide-resistant" type. Most impressive, therefore, is the activity shown against the latter strain by the majority of the anilides. Compounds 11, 15, 17, and 18 were the only derivatives ineffective against strain Spr.

#### *Tests with p-Aminobenzoic Acid*

A compound representative of the sulfanilyl-dibromoanilides (compound 8) and one representative of the benzenesulfon-dibromoanilides (compound 10) were each dissolved in distilled water to give molar concentrations of  $10^{-2}$ ,  $10^{-3}$ , and  $10^{-4}$ . Dilutions of *p*-aminobenzoic acid were prepared in a similar manner. All solutions were autoclaved at 10 pounds for 10 minutes. Each

TABLE 4  
Highest dilution of compound showing antibacterial activity

COMPOUND NO.	MENINGOCOCCUS				GONOCOCCUS*			
	Group I		Group IIA		Strain LED	Strain 91	Strain 66	Strain SPR
	Bs	Bc	Bs	Bc				
1 & 8	128	32	64	32	50	50	50	20
2 & 9	64	32	32	16	50	75	50	20
3 & 10	32	32	32	16	100	150	75	50
4	16	8	8	8	10	10	10	10
5	16	8	8	8	20	10	20	10
6 & 7	32	8	32	8	10	10	10	5
11	8	<1	8	<1	<1	<1	<1	<1
12	32	16	16	16	20	40	20	10
13	16	16	16	16	20	20	20	10
14	32	16	32	16	75	100	50	20
15	16	16	16	16	40	40	20	<1
16	32	32	32	32	75	75	50	20
17	4	<1	8	<1	<1	<1	<1	<1
18	16	<1	16	<1	<1	<1	<1	<1
Sulfanilamide	8	2	8	8	5	10	5	<1
Sulfathiazole	128	16	256	32	20	40	50	<1
Sulfadiazine	128	4	256	2	2	20	40	<1
<i>p</i> -Aminomethylbenzenesulfonamide	8	1	16	8	<1	5	2	<1

See legend under table 2.

\* = Bactericidal activity only.

TABLE 5  
Effect of *p*-aminobenzoic acid upon compounds

TEST COMPOUND MOLAR CONCENTRATION		FABA-MOLAR CONCENTRATION											
		10 <sup>-3</sup>			10 <sup>-4</sup>			10 <sup>-5</sup>			0		
		24	48	72	24	48	72	24	48	72	24	48	72
Sodium salt of sulfanilyl-3,5-dibromoanilide	10 <sup>-3</sup>	0	0	0	0	0	0	0	0	0	0	0	0
	10 <sup>-4</sup>	0	0	4	0	0	4	0	0	4	0	0	4
	10 <sup>-5</sup>	4	4	4	4	4	4	4	4	4	4	4	4
	0	4	4	4	4	4	4	4	4	4	4	4	4
Sodium salt of benzenesulfon-3,5-dibromoanilide	10 <sup>-3</sup>	0	0	0	0	0	0	0	0	0	0	0	0
	10 <sup>-4</sup>	0	2	2	0	2	2	0	2	2	0	2	2
	10 <sup>-5</sup>	4	4	4	4	4	4	4	4	4	4	4	4
	0	4	4	4	4	4	4	4	4	4	4	4	4

0 = No growth.

2 = Approximately half as much growth as control tube.

4 = Growth equivalent to broth control.

concentration of test compound was mixed with each concentration of *p*-aminobenzoic acid in veal glucose broth by aseptically pipetting sterile solutions per tube as follows:

	ml
Veal glucose broth .....	7.8
PABA solution.....	1.0
Solution test compound.....	1.0
Normal horse serum.....	0.1
	9.9

To each tube was added 0.1 ml of a 1:500 dilution of a 24-hour veal glucose broth culture of *Streptococcus pyogenes* (C-203). The final concentrations of *p*-aminobenzoic acid and the test compounds thus became  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$  molar. The tubes were incubated at 37 C and examined for growth after 24, 48, and 72 hours. The data (table 5) show that *p*-aminobenzoic acid, in the concentrations tested, did not antagonize the bacteriostatic activity of either test compound.

#### DISCUSSION

While studying the mechanism of action of sulfanilamide, Woods (1940) noted that small amounts of *p*-aminobenzoic acid would block completely the antibacterial effects of the compound. On the basis of this observation, Woods and Fildes (1940) advanced a theory to explain the mechanism of action of sulfanilamide upon bacteria. These investigators assumed that *p*-aminobenzoic acid is an essential growth factor for the organism and noted that relatively minute amounts of the acid would antagonize the antibacterial effects of sulfanilamide. On the basis of their findings, they explained the interference of sulfonamide action as a competitive inhibition due to the structural relationship existing between the two compounds. Until recently, all sulfonamides containing a free  $\text{NH}_2$  group were found to be antagonized by the presence of *p*-aminobenzoic acid. The fact that *p*-aminomethylbenzenesulfonamide, and later the benzenesulfonanilides, were unaffected by *p*-aminobenzoic acid was anticipated in part, inasmuch as in the former compound the *para*-amino group is separated from the benzene ring by a methyl radical, and in the latter compound the amino group is entirely lacking. Schreus (1942) postulated that whereas *p*-aminobenzoic acid is not concerned with the action of *p*-aminomethylbenzenesulfonamide, one of its analogues, namely, *p*-aminomethylbenzoic acid, is the metabolite which is in competition with *p*-aminomethylbenzenesulfonamide for the growth of the organisms. One of us (Lawrence, 1944, 1945), however, has brought forth evidence of the inability of *p*-aminomethylbenzoic acid to interfere with the antibacterial effects of *p*-aminomethylbenzenesulfonamide, thus invalidating the theory of Schreus and proving this relationship to be incompatible with the Woods-Fildes theory.

Contrary to expectation, the sulfanilylanilides studied in the present investigations, although containing a free  $\text{NH}_2$  group in the *para*-position, were un-

affected by the presence of *p*-aminobenzoic acid. The results obtained both with the sulfanilylanilides and with *p*-aminomethylbenzenesulfonamide, therefore, are in contradiction to the tenets of the Woods-Fildes theory.

Probably one of the best approaches to the solution of the problem of the mechanism of sulfonamide action is a study of drug antagonists. The antagonism displayed by *p*-aminobenzoic acid toward the commonly known sulfonamides (sulfanilamide, sulfathiazole, etc.) suggests that antibacterial activity is produced by interference with some metabolic function of the bacterial cell. We have tested a large number of vitamins and vitamin derivatives, amino acids, and carbohydrates for a possible antagonistic effect toward the compounds described in this report, but as yet none have shown any evidence of this activity.

#### SUMMARY

The *in vitro* antibacterial effects of a series of benzene sulfonic acid derivatives are presented.

The compounds which have halogen groups substituted in the 3,4 or 3,5 positions on the anilide portion of the molecule are usually more effective than the mono-halogenated compounds.

An acetyl group in the N<sup>4</sup> position greatly diminishes the antibacterial effects of the parent compound.

The sulfanilylanilides and benzenesulfonanilides are unaffected by the presence of *p*-aminobenzoic acid.

#### ACKNOWLEDGMENT

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# ANTIFUNGAL PROPERTIES OF ANTIBIOTIC SUBSTANCES<sup>1</sup>

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## INTRODUCTORY

Fungi, especially the filamentous types, have become definitely established as the most important group of organisms that is capable of producing antibacterial substances under proper conditions of culture. No less significant, even if less well known, is the fact that many microorganisms, including fungi on the one hand, and bacteria and actinomycetes on the other, are capable of producing agents which possess antifungal properties. Some of the antibiotic substances which are active against bacteria are also characterized by fungistatic and fungicidal activities; others, however, may be active upon bacteria but not upon fungi.

These facts have long been known to the plant pathologists, who were impressed by the ability of fungi to live in close association with other microorganisms in natural substrates, especially in the soil. Many of the organisms antagonistic to fungi were known to have a specific capacity of inhibiting the growth of various plant-pathogenic fungi and even of causing their destruction. This effect is selective and varies for different fungi, some being affected greatly and others slightly or not at all. The practical utilization of this phenomenon for the control of plant diseases has, therefore, been suggested (Chudiakov, 1935; Novogrudsky, 1936) and has actually been utilized on a limited scale. However, the ability of antagonistic microorganisms to attack fungi causing animal diseases (Chambers and Weidman, 1928) and the utilization of the antibiotic substances produced by these organisms for the purpose of combating such pathogens have received only cursory consideration (Waksman, 1945).

The favorable effects that resulted from the practical utilization of certain antibiotic substances, such as tyrothricin, penicillin, and streptomycin, for the control of various human and animal diseases caused by bacteria suggested the possibility that some of these agents may also be utilized for the control of human and animal diseases caused by fungi.

No attempt will be made to review here the very extensive literature on the antagonistic effects of various microorganisms upon fungi (Waksman, 1941). Studies of these reactions have resulted in the isolation of crystalline gliotoxin (Weindling, 1934) and in the demonstration that various bacteria produce antifungal agents (Chudiakov, 1935; Cordon and Haenseler, 1939).

The effect of antibiotic substances upon fungi resulted in a modification of the morphology of the organisms, a change in some physiological mechanism such

<sup>1</sup> Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.



as pigment production or growth, or in a complete destruction of the organism. Fungi treated with certain yeasts, for example, produced thick gnarled mycelia, without any conidia or pigment (Cook *et al.*, 1941). The mechanism of disintegration of the hyphae of the plant-pathogenic fungus *Rhizoctonia* by the antagonistic fungus *Trichoderma*, as well as by its specific agent gliotoxin, was studied in detail by Weindling (1934). The hyphae of the *Rhizoctonia* were killed in less than 10 hours, as was shown by a loss of the homogeneous appearance of the protoplasm and the vacuolate structure of the hyphae.

Stokes *et al.* (1942) reported that the antibacterial agents, pyocyanine, hemipyocyanine, and tyrothricin, also possess marked fungistatic properties. The fungi affected included the pathogenic forms *Achorion schoenleinii*, *Trichophyton gypseum*, *Microsporum gypseum*, and *Candida albicans*, the first of these being the most sensitive, and the last the most resistant. Tyrothricin inhibited the growth of all four fungi in dilutions of 1:5,000 to 1:20,000. Pyocyanine was the least active of the three compounds, and hemipyocyanine the most active. Among the other antibiotic agents found to inhibit the growth of fungi, it is sufficient to mention actinomycin and clavacin. The ability of actinomycin to affect the growth of *Ceratostomella ulmi* could be partly inhibited or neutralized by the addition of pyridoxine, thus pointing to the possible mechanism of the fungistatic action of actinomycin (Waksman and Bugie, 1943). The marked fungistatic properties of clavacin have also been indicated (Waksman, Horning, and Spencer, 1942, 1943).

In a study of the antifungal properties of streptothricin and streptomycin, two closely related antibiotic substances produced by actinomycetes, Robinson, Smith, and Graessle (1944) demonstrated that whereas streptothricin has considerable activity against both pathogenic and saprophytic fungi, streptomycin has very little effect against these organisms.

A comparison has been made (Geiger and Conn, 1945) of the bacteriostatic and fungistatic properties of certain antibiotic substances, notably penicillic acid and clavacin, that possess chemical properties similar to ketones. The first of these had only slight antifungal activity, but the second was very active. Certain synthetic unsaturated ketones were found to have even greater antifungal properties than clavacin.

#### EXPERIMENTAL

Different fungi are known to vary greatly in their sensitivity to the same antibiotic substance; it has also been established that there is a marked variation in the activity of different substances upon the same organism. The purpose of this investigation was to compare the action of various antibiotic substances upon several fungi, in order to justify certain broad generalizations. In an attempt to evaluate an antibiotic substance from the point of view of its possible use as a chemotherapeutic agent, special consideration is also given to the toxicity of the substance to animals. A more toxic substance, even if it is characterized by greater antifungal activities, may offer less promise as a chemotherapeutic agent than another substance which is less toxic and also less active.

*Methods.* Two methods were used for the evaluation of the fungistatic effect and the antifungal spectrum of an antibiotic substance: (1) the agar streak or agar dilution method, and (2) the agar diffusion or cup method.

In the agar streak method the antibiotic substance is added, in different concentrations, to the agar medium placed in ordinary sterile petri dishes. Several, usually 2 to 5, test organisms are streaked on each plate. The plates are incubated, at 28 C, or 37 C, for 24 to 48 hours and records made. The

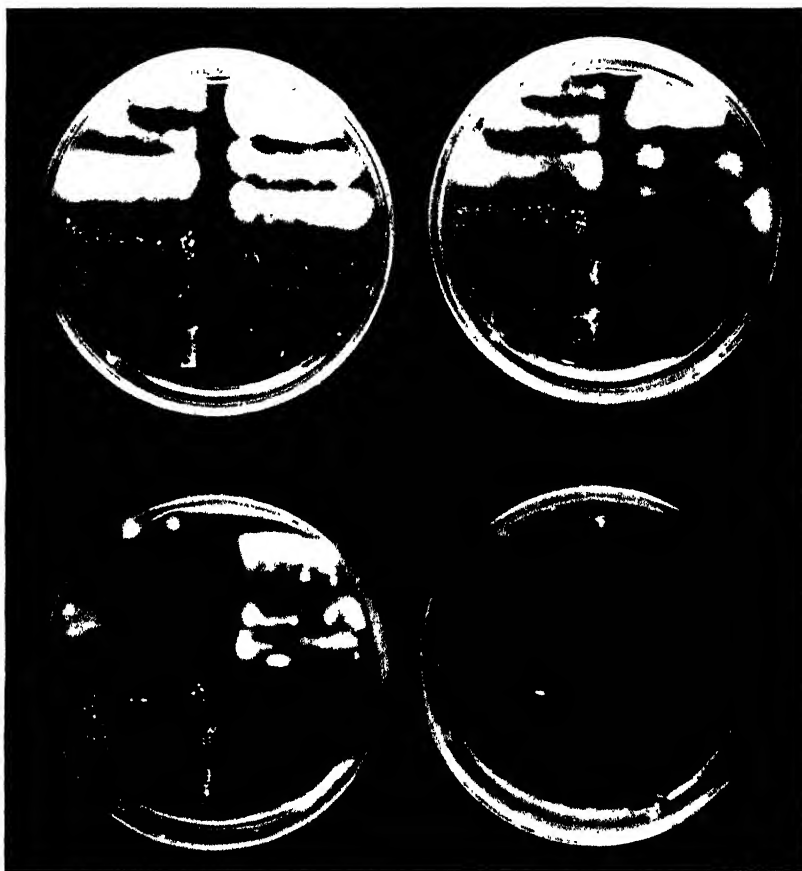


FIG. 1. SENSITIVITY OF DIFFERENT FUNGI TO AN ANTIBIOTIC SUBSTANCE, AS ILLUSTRATED BY THE AGAR STREAK METHOD

amount of the substances required to inhibit completely the growth of each test organism is taken as the end point; the activity of the preparation is calculated on a unit per milliliter basis for one gram of the material. The ratio of the volume of agar to the amount of the substance required to inhibit a given test organism gives the number of antifungal units of a substance. Figure 1 shows that fungi vary in their sensitivity to the same substance, since they are inhibited by different concentrations of the substance.

In the cup method the agar medium is inoculated with a single test organism,

and different dilutions of the substance are placed in several cups, partly inserted into the agar. The zone of inhibition of growth of the test fungus is taken as a measure of the activity of the substance as compared with that of a standard preparation. Figure 2 shows that there is a direct proportion between the concentration of the active material and the zone of the diffusion. Attention may be directed here to the two zones in each ring. The outer zone is the one that

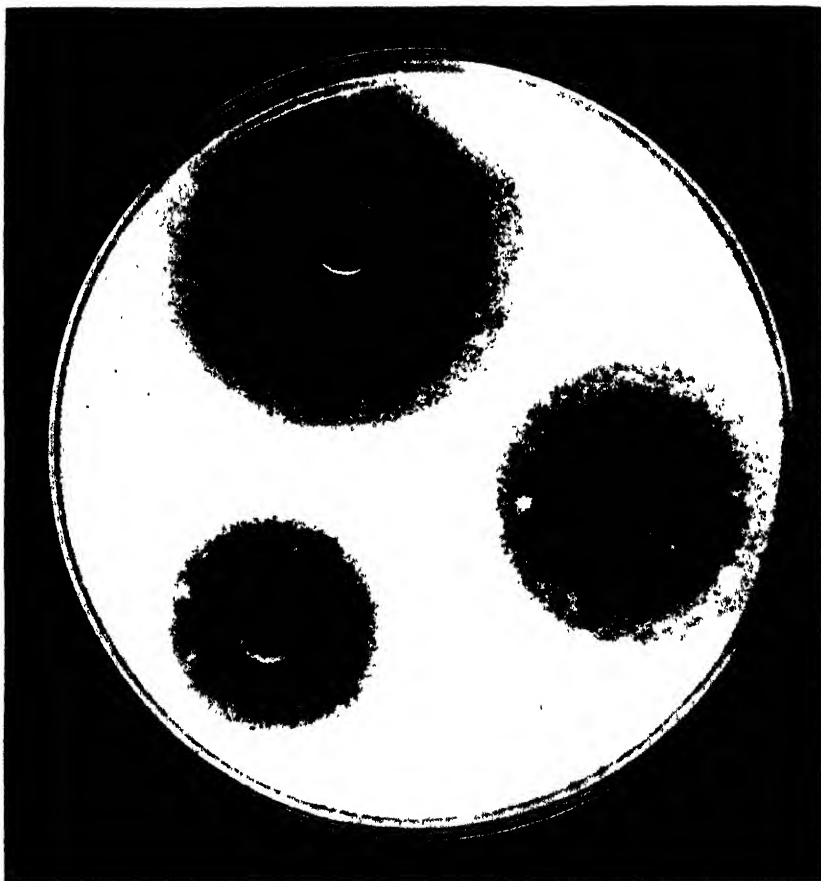


FIG. 2. CUP METHOD FOR TESTING THE FUNGISTATIC ACTION OF AN ANTIBIOTIC SUBSTANCE, AS SHOWN BY THE EFFECT OF STREPTOTHRICIN UPON *TRICHOPHYTON MENTAGROPHYTES*

The dilutions used are 1, 1:5, and 1:25

is measured. The inner zone is due to the overgrowth by the surface mycelium of some portion of the inhibited zone. Certain fungi, such as the pathogen *Trichophyton mentagrophytes*, can be readily utilized for assaying the fungistatic potency of various substances (Emmons, 1944) in a manner similar to the use of *Staphylococcus aureus* or *Bacillus subtilis* for testing the antibacterial potency of antibiotic substances. The results can also be expressed in dilution units by standardizing the results of the cup method against the agar streak method with the same organism.

*Test fungi.* Four fungi pathogenic to animals and to man and four saprophytes were used in this study. The first group included *Candida albicans*, *Trichophyton mentagrophytes* 589, *Trichophyton mentagrophytes* 640, and *Cryptococcus neoformans*; the second group comprised a strain of *Penicillium luteum-purpurogenum*, *Dematium*, *Fusarium*, and *Aspergillus clavatus*.

In carrying out the tests, two different media were used, namely, fungous (glucose peptone salts) agar and ordinary nutrient (meat-extract peptone salt) agar.



FIG. 3. METHOD OF TESTING THE PRODUCTION OF AN ANTIFUNGAL SUBSTANCE BY A FRESHLY ISOLATED CULTURE

In the isolation from natural substrates of organisms that have the capacity of producing fungistatic substances, the agar streak method, similar to the one used in the testing of organisms producing substances active against bacteria (Waksman and Horning, 1943) was used. The method is illustrated in figure 3.

*Antibiotic substances.* Several antibiotic substances were used in this study. These substances varied greatly in chemical nature, in biological activity, and in the degree of their purification. Some, like actinomycin, fumigacin, gliotoxin, and clavacin, were crystalline preparations; others, like chaetomin, strepto-

thricin, and streptomycin, were highly purified but not as yet crystallized. The antibacterial activity of each substance can serve as a relative measure of its respective antibiotic potency.

*Fungistatic action of antibiotic substances.* In the following studies, known antibiotic substances have been used. The results obtained are presented in tables 1 and 2. The various substances used in these studies can be arranged, on the basis of their relative fungistatic activity, in the following order, beginning with the most potent: gliotoxin, actinomycin, clavacin, fumigacin, streptothricin, chaetomin, and streptomycin.

Gliotoxin is fairly toxic to animals, but because of its extremely high fungistatic properties it deserves more detailed consideration. The antifungal

TABLE 1  
*Fungistatic action of antibiotic substances on saprophytic fungi*  
Activity, units per gram\*

ANTIBIOTIC SUBSTANCE	ANTIBACTERIAL ACTIVITY		PENICILLIUM TUTTIUM PURPURA- GENUM	DEMATIUM SP	FUSARIUM SP	ASPERGILLUS CLAVATUS 14a
	<i>E. coli</i>	<i>B. subtilis</i>				
Fungistatic activity, using fungus agar						
Actinomycin		>20,000,000	<500,000	<500,000	<500,000	<500,000
Chaetomin		750,000,000	<4,000	<4,000	<4,000	<4,000
Clavacin	100,000	200,000	25,000	15,000	25,000	3,000
Fumigacin	7,200	600,000	8,900	22,000	22,000	3,000
Gliotoxin	15,000	2,000,000	600,000	6,000,000	600,000	200,000
Streptomycin	125,000	625,000	<45	<45	<45	<45
Streptothricin	100,000	500,000	3,000	6,000	9,000	<60
Fungistatic activity, using nutrient agar						
Streptomycin	125,000	625,000	<120	<120	<120	<120
Streptothricin	100,000	500,000	7,100	29,000	4,800	2,900

\* 24 hours' incubation at 28 C.

activities of this substance vary greatly, quantitatively, with respect to the pathogenic as well as the saprophytic fungi.

Actinomycin and clavacin are highly toxic to animals, and the second is not characterized by a very high activity as compared with gliotoxin. Both of these substances can, therefore, be eliminated from further consideration as fungistatic agents offering promise for chemotherapeutic purposes.

The fumigacin used in these experiments was a crude preparation, having a slight admixture of gliotoxin, which may account for its limited antifungal properties. Therefore, fumigacin can hardly be considered as offering much promise for practical utilization.

Chaetomin and streptomycin were completely inactive against fungi and can, therefore, also be eliminated from further consideration.

Streptothricin was found to possess definite fungistatic properties, and, since

this is not a very toxic compound, it should be considered further. It is of particular interest to note that the two basic preparations used in this study, streptothricin and streptomycin, both of which possess similar chemical and antibacterial properties, differ greatly in their antifungal activities: the second is completely inactive, whereas the first has some definite activity against fungi. These observations thus tend to confirm those obtained by Robinson, Smith, and Graessle (1944).

The results of a survey of seven different antibiotic substances brought out the fact that only two of these, gliotoxin and streptothricin, merit consideration for possible practical application. They were therefore selected for further study.

TABLE 2

*Fungistatic action of antibiotic substances—pathogenic fungi*

Activity, units per gram\*

ANTIBIOTIC SUBSTANCE†	CANDIDA ALBICANS	TRICHOPHYTON MENTAGROPHYTES 598	TRICHOPHYTON MENTAGROPHYTES 640	CRYPTOCOCCUS NEOFORMANS
Fungistatic activity, using fungus agar				
Actinomycin .....	<500,000	5,000,000	1,500,000	1,500,000
Chaetomin .....	<4,000	<4,000	<4,000	<4,000
Clavacin .....	3,300	33,000	25,000	10,000
Fumigacin.....	8,900	14,800	8,900	30,000
Gliotoxin.....	4,000,000	6,000,000	2,000,000	>20,000,000
Streptomycin.....	<45	<45	<45	<45
Streptothricin..	<60	4,500	4,500	12,000
Fungistatic activity, using nutrient agar				
Streptomycin... ..	<120	<120	<120	<120
Streptothricin.....	1,900	2,900	2,900	19,000

\* 2 to 3 days' incubation at 28 C.

† The antibacterial activity of these preparations is given in table 1.

*Fungicidal action of antibiotic substances.* The fungicidal action of antibiotic substances was determined as follows: 5-ml portions of nutrient broth were placed in 50-ml Erlenmeyer flasks and inoculated with 2 drops of a sterile water suspension of the spores of the test fungus. The cultures were incubated, with frequent shaking, at 37 C for 1 to 3 days. The antibiotic agent was added in different concentrations. The cultures were further incubated and streaked on nutrient agar plates after 1, 3, and 7 days. The plates were then incubated at 37 C for 48 hours, and readings taken.

In the first experiment, 3 antibiotic agents were used: fumigacin, 33.8 mg per ml; gliotoxin, 5 mg per ml; and streptothricin (100 units per mg), 100 mg per ml. The results obtained are presented in table 3.

Gliotoxin proved to be the most active fungicidal agent; when too low con-

centrations were used, there appeared to be a definite adaptation of the organism to the antibiotic substance, and an early destructive effect was later overcome. In the case of streptothricin, however, the fungicidal action appeared to be greater on continued contact of the agent with the fungus.

The results of an experiment on the antifungal action of clavacin and synthetic ketones are presented in table 4. Acrylophenone was the most active agent. The antifungal properties of this phenone were compared with those of the two antibiotic substances, gliotoxin and streptothricin. The test fungi were grown in nutrient broth for 1 and 2 days, and the active substances added (table 5). Acrylophenone proved to be most actively fungicidal, followed by gliotoxin. Streptothricin was the least active of the three.

TABLE 3  
*Fungicidal action of antibiotic substances*

ANTIBIOTIC AGENT	MG PER 5 ML CULTURE	<i>C. albicans</i> 3147			<i>T. mentagrophytes</i> 598		
		1 day	3 days	7 days	1 day	3 days	7 days
Fumigacin . . . . .	3.4	0+	0	0	0	0	0
Fumigacin . . . . .	0.7	trace	+++	+++	trace	0	0
Gliotoxin . . . . .	2.5	0	0	0	0	0	0
Gliotoxin . . . . .	0.5	0	+	+++	0	0	0
Gliotoxin . . . . .	0.1	+	+++	+++	0	0	trace
Streptothricin . . . . .	50.0	+++	++	trace	++	+	+
Streptothricin . . . . .	10.0	+++	+++	+	+++	+++	++
Alcohol 95%* . . . . .	0.5 ml	+++	+	+++	+++	0	+
Alcohol 95%* . . . . .	0.1 ml	+++	+++	+++	+++	+++	+++
Control . . . . .		+++	+++	+++	+++	+++	+++

+0 = no growth; + = limited growth; ++ = medium growth; +++ = maximum growth.

\* The fungicidal activity of 95% alcohol was investigated because the fumigacin and gliotoxin were in 95% alcoholic solution.

The antifungal properties of antagonistic organisms and of antibiotic substances involve three distinct types of effect: (1) a lytic action, whereby the hyphae and the spores of the fungus are dissolved, the organism losing completely its ability to reproduce; (2) a fungicidal action, which is not necessarily accompanied by lysis; (3) a fungistatic effect, the fungus not being killed, but being prevented from growing. The latter effect may be accompanied by an abnormal development of the hyphae, which do not, however, lose the power of growth and reproduction when placed under favorable conditions of culture. A strong fungistatic effect, combined with limited injury to the tissues of the host, appears to be most significant in any consideration of the chemotherapeutic potentialities of a substance.

TABLE 4

*Fungistatic action of unsaturated ketones on pathogenic fungi*

SUBSTANCE	DILUTION UNITS PER GRAM OF SUBSTANCE			
	<i>Cryptococcus neoformans</i>	<i>Trichophyton mentagrophytes</i> 640	<i>Trichophyton mentagrophytes</i> 598	<i>Candida albicans</i>
Acrylophenone.....	30,000	>3,000,000	>3,000,000	30,000
Benzalacetone.. ..	16,000	160,000	160,000	20,000
Benzalacetophenone.....	60,000	>200,000	>200,000	20,000
Furfuralacetophenone....	6,000	16,000	20,000	2,000
Clavacin. ....	30,000	30,000	50,000	<10,000

TABLE 5

*Fungicidal action of antibiotic substances and of a synthetic phenone*

ANTIBIOTIC SUBSTANCE	MG PER 5 ML BROTH	1-DAY-OLD CULTURES VIABILITY AFTER TREATMENT FOR		2-DAY-OLD CULTURES VIABILITY AFTER TREATMENT FOR	
		2 days	5 days	2 days	5 days
<i>Candida albicans</i> 3147					
Gliotoxin. ....	0.5	tr	+++	tr	tr
Gliotoxin. ....	0.1	+++	+++	+++	+++
Acrylophenone. ....	1.0	0	0	0	0
Acrylophenone. ....	0.2	+++	+++	+++	+++
Acrylophenone. ....	0.04	+++	+++	+++	+++
Control. ....	0	+++	+++	+++	+++
<i>Trichophyton mentagrophytes</i> 598					
Gliotoxin. ....	0.5	0	0	0	0
Gliotoxin. ....	0.1	tr	tr	tr	tr
Acrylophenone. ....	1.0	0	0	0	0
Acrylophenone. ....	0.2	0	0	0	0
Acrylophenone. ....	0.04	+	tr	+++	+++
Streptothricin. ....	50.0	tr	+	+	+
Streptothricin. ....	10.0	tr	+	+++	+++
Control. ....	0	+++	+++	+++	+++

## SUMMARY

From the results of a study of the antifungal action of antibiotic substances produced by microorganisms and of certain synthetic compounds, the following conclusions may be drawn:

(1) Antibiotic substances vary greatly in their antifungal effect. Some, like gliotoxin and actinomycin, are very active, and others, like chaetomin and streptomycin, have very little activity.



(2) The selection of an antibiotic substance for chemotherapeutic purposes depends not only upon the relative activity of the substance but also upon its toxicity to animals. Hence, a substance like actinomycin, which is highly active but is also highly toxic, is eliminated from practical consideration, whereas a substance like streptothricin, which is not so active but also is not very toxic, deserves consideration.

(3) Of seven antibiotic substances tested for their antifungal properties, only two were found worthy of consideration for practical utilization, gliotoxin and streptothricin.

(4) The antifungal action of an antibiotic substance comprises both fungistatic and fungicidal effects.

(5) Certain unsaturated ketones, to which some of the antibiotic substances belong, were found to possess very strong antifungal properties.

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# ACTIVE ENZYME PREPARATIONS FROM BACTERIA

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For many years attempts have been made to obtain cell-free enzyme preparations from bacteria in order to extend our knowledge of their metabolism. Werkman and Wood (1940) reviewed many of those attempts. The methods, generally, were too limited in scope and results could not be duplicated easily, or treatment was either inadequate or too drastic. No satisfactory method for obtaining cell-free enzyme preparations from bacteria was available until Booth and Green (1938) developed their stainless steel mill. By the use of this mill, preparations were obtained from various species of yeast, *Sarcina lutea*, *Bacillus subtilis*, and *Escherichia coli*. Despite the success of the Booth-Green mill, it has two serious disadvantages: (1) expense and (2) use of metal surfaces for grinding.

Wiggert *et al.* (1940) developed a method of grinding bacteria with powdered glass with equipment which was readily available, i.e., a ball mill to powder the glass, and a mortar and pestle to grind the cell-glass mixture and thus disrupt the cells. Using this technique, they obtained active preparations from *E. coli*, *Aerobacter aerogenes*, *Citrobacter freundii*, and species of *Clostridium*. The disadvantage of this method was that the grinding of the bacteria with mortar and pestle was done by hand. Thus the product was not uniform, and preparations varying in activity were obtained. The method has, therefore, been further improved. It is the purpose of this communication to describe an adaptation whereby the bacteria, mixed with glass, are ground by being gently forced between two close-fitting, concentric, ground-glass cones, the inner one of which is rotated. This gives more uniform preparations of much greater activity.

## METHOD

### *Growth and Harvesting of Organisms*

Large quantities of bacterial cells are required. Generally, 10 to 15 grams of cells (wet weight) are sufficient. With most organisms these amounts may be obtained by inoculating the organism into 10 liters of a suitable nutritive medium contained in a 12-liter pyrex flask. In this manner, depending on the organism and the medium used, 1.5 to 4.0 grams of wet cells are usually obtained per liter of medium. Satisfactory yields can also be obtained by growing the cells aerobically, e.g., on agar. However, cells grown on a solid agar medium seem more resistant to grinding and yield weaker enzyme preparations than those grown in a liquid medium. For aerobic growth in liquid media, air is filtered through cotton, conducted through glass tubing into the medium, and then passed through a fine, porous, gas-dispersing stone at the bottom of

the medium. To prevent excessive foaming on strong aeration, a small amount of octadecyl alcohol, or paraffin, may be added to the medium before sterilization.

The medium employed will of course vary with the nutritive requirements of the particular organism. The following medium has consistently yielded good crops of cells with coliform organisms: 1 per cent glucose (corn or grape sugar), 0.8 per cent  $K_2HPO_4$  (technical grade), 0.4 per cent Bacto peptone or yeast extract, and about 10 per cent tap water (if distilled water is used as diluent). The medium is sterilized at 15 pounds' pressure. The solution of phosphate in tap water is sterilized separately. This procedure decreases the caramelization of the sugar.

The inoculated medium is generally incubated for 18 to 24 hours; thus a good crop of relatively young, active cells is obtained. Young cells are to be preferred since they are more active. The cells are harvested by centrifugation in a Sharples supercentrifuge run at about 35,000 revolutions per minute. Cells which are allowed to grow for 36 and even 48 hours still yield active preparations on grinding. The cells may be washed and recentrifuged, if desired. In most cases washing is not necessary, since the endogenous activity of the bacterial juices obtained by grinding is negligible.

The wet bacterial cell paste may be stored for 24 hours if kept in a humid atmosphere at low temperature.

### *Mixing with Glass*

Small pieces of pyrex glass are cleaned, dried, and placed in a ball mill for powdering. With large pebbles or stones, 24 hours usually suffices for proper powdering. If the mill contains steel balls, the powdering is generally accomplished within 1 to 4 hours. With new steel balls the first two or three batches of ground glass may have a slight grayish color, and may therefore have to be discarded. After that, no trouble is encountered.

The powdered glass is then passed through a set of sieves which prevent glass particles passing larger than 0.5 mm in diameter. The average size of the glass particles should be about 2 microns. It is advisable to wear a dust mask during the transfer of the glass from the ball mill to the sieves and also during the sifting process.

The cell paste (as obtained from the Sharples centrifuge) is mixed with the powdered glass. To every gram of wet paste 2 grams of ground glass are added and the resulting glass-paste is mixed thoroughly until it resembles a rather firm batter. The consistency of the mixture is important; if the glass-paste mixture is too fluid, the grinding process will be inefficient and the resulting enzyme preparations weak. On the other hand, if the mixture is too stiff, it may slow down the rate of rotation of the inner cone or stop it altogether. It is difficult to describe the correct consistency of the glass-paste mixture, but after thorough mixing with a spatula the paste should show the path of the spatula for a short time but not permanently. To thicken the mixture add a small amount of glass, or to thin it add several drops of distilled water or phosphate buffer, and mix thoroughly. The ratio of two parts of glass to one of paste can be raised somewhat without reducing the activity.

Several experiments were carried out to determine the effect of varying the ratio of paste to glass on the activity of the enzyme. Ratios of wet bacterial cells to glass of 1:8, 1:5, 1:3, and 1:2 were tried. It was found that the activity of the enzyme obtained after grinding increased as the proportion of glass decreased in the order listed, and the optimum ratio for grinding was found to be 1:2 of bacterial cell paste to powdered glass.

Experiments were also conducted to determine the comparative values of glass and carborundum (alloxite brand powder, no. 1000 and finer) as grinding agents in obtaining active preparations. Generally, under several sets of conditions, powdered glass was found to be superior in yielding active preparations.

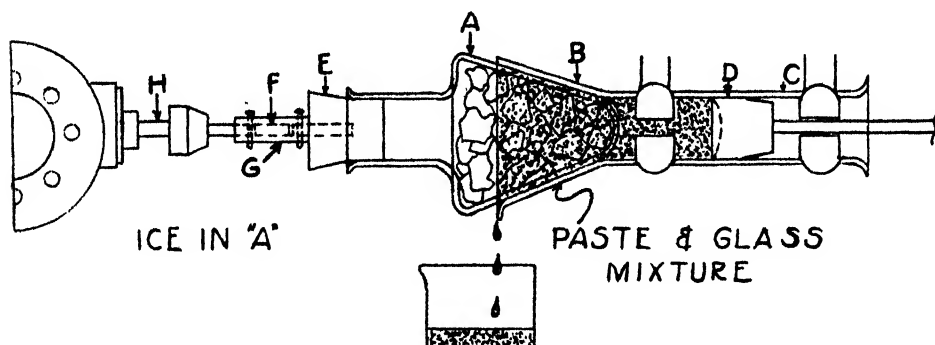


FIG. 1. BACTERIA GRINDER

- A = Inner cone
- B = Standard taper glass cone
- C = Glass tube
- D = Plunger
- E = Rubber stopper
- F = Metal tubing
- G = Rubber tubing
- H = Motor rotating shaft

### *Description of Apparatus and Process*

The grinder consists of two close-fitting, concentric, ground-glass cones mounted in a horizontal position (figure 1). The inner cone (A) is rotated steadily by an attachment to a motor which is geared down to approximately 150 revolutions per minute, whereas the outer cone (B) is held in a fixed position. The motor must be rather powerful, so that the shaft cannot be easily stopped with the hand. A flexible connection between the inner cone (A) and the horizontal rotating shaft of the motor (H) is desirable to achieve intimate contact between the two ground-glass surfaces with much less danger of breakage. This flexible connection is obtained by inserting a short piece of rubber tubing (G) over the rotating shaft of the motor and wiring it tightly in place. A short piece of metal tubing (F) serves as the connection between the rubber tubing (G) and the rubber stopper (E) inserted in the open end of the inner ground-glass cone (A). If (F) contains a small hole near each end, the connections between (G) and (F) and (E) may be made secure by forcing a nail through the

inserted parts. The outer cone consists of a glass tube (C) sealed to a standard taper glass cone (B) which is ground to fit the inner cone (A).<sup>1</sup>

The glass-paste mixture is inserted in the open end of (C), and the tube is filled. The inner cone is filled with crushed ice and attached to the motor. The inner and outer cones are then joined, the motor started, and the cell-glass paste is slowly forced between the rotating, ground-glass cones by means of a plunger (D) fashioned from a rubber stopper. A slight but steady pressure with the hand against the fixed cone forces the grinding surfaces more closely together and aids the grinding process. The speed of the motor is conveniently controlled by means of a rheostat. The extruded material is caught in a beaker, surrounded by crushed ice, directly underneath.

The cell-glass paste may be ground twice, if necessary. It may be necessary to add a small amount of glass at the end of the first grinding. Extended grinding of the bacterial cells should be avoided, for this results in inactivation.

The ground material, kept cold, is extracted by thoroughly mixing it with cold dilute phosphate buffer. In some cases distilled water has been successfully used. One and five-tenths milliliters, or less, of buffer or water are added for every gram of bacterial paste originally employed. The extraction time does not have to be extended; after thorough mixing, ten minutes of contact between the ground material and the extract buffer are generally sufficient before centrifugation is started.

### *Centrifugation*

The glass is thrown down by centrifugation for five minutes on an International or angle centrifuge at approximately 3,500 revolutions per minute. The supernatant liquid is further clarified by centrifuging the cells and cell particles in an air-driven Beams "spinning top" ultracentrifuge (Beams, 1930; Beams *et al.*, 1933). This centrifuge can be constructed at low cost by any competent instrument maker. The centrifugal force developed depends largely on the rotor diameter and the pressure of air used for driving the rotor. With a rotor of approximately 3-cm diameter containing 4.5 ml of liquid at an air pressure of 60 to 64 pounds, the centrifuge reaches an estimated speed of 75,000 to 100,000 revolutions per minute. The cell fragments are deposited on the wall of the rotating cup, and the clear liquid is removed from the center of the cup by means of a capillary pipette. With most preparations centrifugation for 10 minutes yields a clear juice. The centrifugation period varies from 5 to 30 minutes, depending largely on the degree of clarification desired and the liquid used in extraction. In general, water extracts clear more slowly than phosphate extracts. A Beams centrifuge is not essential for the production of the juice. With reasonable care in cooling, any good centrifuge will sufficiently clarify most of the bacterial extracts. It should be emphasized that the bacterial preparation should be kept ice-cold at all times after the grinding process is completed. In

<sup>1</sup> Cones of thick glass, built to specifications from standard taper joints, are obtainable from the Scientific Glass Company, Bloomfield, New Jersey.

addition, it is desirable to complete the centrifugation of the juice immediately after extraction.

The final bacterial extract is a clear, yellow-brown liquid, slightly viscous and opalescent.

#### EXPERIMENTAL

##### *Cell Counts of Enzyme Preparations*

The preparation is not entirely free of bacterial cells because of difficulties of removing the juice from the centrifuge cup. Since in most cases the preparation contains considerable numbers of viable cells, it is necessary to determine (1) the number of viable cells able to cause appreciable activity under standard

TABLE 1

*Numbers of viable organisms necessary to cause acid and gas production on respirometer*

NUMBER OF ORGANISMS (PLATE COUNT) X 10 <sup>6</sup>	MICROLITERS CO <sub>2</sub> EVOLVED IN				
	0-30 min	30-60 min	60-90 min	90-120 min	120-150 min
none	32	20	20	16	17
375.0	27	213	312	263	147
187.5	26	125	173	236	220
93.75	40	48	53	75	68
46.88	26	37	45	54	56
23.24	30	23	23	24	24
11.62	35	23	34	31	31

Juice prepared from *Aerobacter indologenes*. Suspension of unground cells made up to various dilutions and added as indicated. Each vessel contained bacterial juice, 0.6 ml; glucose, 0.055 M; hexosediphosphate, 0.03 M; NaHCO<sub>3</sub>, 0.05 M; total volume, 2.0 ml; atmosphere, CO<sub>2</sub>; temperature, 30.4 C.

conditions and (2) the part of the activity of the bacterial juice caused by the presence of living bacterial cells.

A juice was prepared from *A. indologenes* to which were added known numbers of organisms to determine the least number causing an increase in activity. The juice was added to make conditions analogous to those actually encountered. An aliquot of the suspension was plated on nutrient agar and the approximate number of viable cells added to the Warburg vessel was determined. The experiments of table 1 show that approximately 45 million cells were required per cup (containing 2.0 ml total volume) before an appreciable increase in activity was noted in 150 minutes on the respirometer under the experimental conditions. It will be noted that in all cases when an effect is observed, the activity is shown to increase progressively. In no case has this effect been apparent with the bacterial enzyme preparation.

To determine the effect of numbers of bacteria present on the activity of the enzyme preparation, a juice obtained from *E. coli* was given varying centrifugal treatment and then tested for activity and the number of viable cells determined.

It was found that two different batches of the same enzyme preparation containing from 198,000 to 270,000 and from 12,000 to 24,000 organisms per 0.6 ml, respectively, had very similar activity when tested on the respirometer (table 2).

Since approximately 45 million cells had to be added to effect an appreciable increase in activity on the respirometer (table 1), it is apparent that the activity of the enzyme preparation was due to the cell-free material present. Furthermore, with careful handling the cell count of the juice can be kept well below 50,000 per ml. The slight decrease in the activity of the juice on extended centrifugation was probably due to the removal of additional particles and debris from the liquid.

It has been determined previously (Wiggert *et al.*, 1940) that filtration of the juice through Seitz, Chamberland, or Jena glass filters resulted in complete

TABLE 2  
*Effect of number of bacteria on activity of enzyme preparation*

ACTIVITY $\mu\text{L CO}_2$ PER HR	AVERAGE $\mu\text{L CO}_2$ PER HR	ORGANISMS PER 0.6 ML OF JUICE
1010	1060	198,000-270,000
1110		
1060		
936	1060	12,000- 24,000
1028		
1012		
868	847	1,800- 18,000
827		

Juice prepared from *Escherichia coli*. Organisms determined by plate count, after suitable dilution of juice. Each vessel contained bacterial juice, 0.6 ml; glucose, 0.04 M; hexosediphosphate, 0.03 M;  $\text{NaHCO}_3$ , 0.06 M; total volume, 2.0 ml; atmosphere,  $\text{CO}_2$ ; time, 1 hour; temperature 30.4 C.

inactivation of the preparation. The behavior of the glass-ground juice on filtration has not been studied; however, Lee *et al.* (1942) report that they were able to pass an *Azotobacter* extract, prepared in this manner, through Berkefeld N or Mandler 15 filters.

#### *Effects of Treatment on Activity of Preparation*

The enzyme extract can generally be kept frozen for 4 or 5 days with no appreciable loss of activity, and for a period of weeks with only gradual loss of activity. In many cases, it was found feasible to dry the bacterial juice by placing the extract in a flat dish in a desiccator containing  $\text{CaCl}_2$  and applying a high vacuum. This method furnishes a product which can be conveniently stored and used as needed. The powder may be stored for at least 4 weeks without appreciable loss of activity, at least with regard to the enzymes concerned in the Embden-Meyerhof-Parnas scheme of carbohydrate dissimilation. The enzymes concerned in the anaerobic dissimilation of pyruvate are completely inactivated on drying. When the extract is frozen prior to drying, however, there is no

loss of activity in preparations tested after 24 hours. The enzymes formic dehydrogenase and hydrogenase present in extracts of *E. coli* are relatively stable. They can be reduced to a powder by freezing and drying *in vacuo* and resuspended in water with no appreciable loss in activity. After having been kept in the dried form for almost two months, these two enzymes still retained the greater part of their activity.

Other methods of obtaining a dry powder were less successful. Precipitation with acetone or ammonium sulfate gave powders with much reduced activity when tested on pyruvate or glucose without further addition of coenzymes or other substances which might have been removed by the treatment.

#### DISCUSSION

The method described has been used successfully with more than ten bacterial species and seems very generally applicable. No attempts have been made to apply the method to the study of pathogens but a few simple changes in the apparatus should make this use feasible.

In application of this method to the preparation of extracts from as yet untried bacterial species, it cannot be overemphasized that constant experimentation will be required to obtain successful results. In particular, the conditions of growth of the cells are important, e.g., age, medium, and temperature. Best results have been obtained with young cultures, and it seems probable that a maximum crop of cells will not yield so active preparations as those from cultures which are still growing.

Several improvements could easily be made in the construction of the glass mill. The method of introducing the cell-glass paste is somewhat inconvenient and could be improved by introduction of a geared pressure feed. The method of obtaining pressure upon the cones by using the hand during the grinding process could be improved by the introduction of a pressure system which would exert a constant force.

#### SUMMARY

A semimechanical adaptation of the glass-grinding method for the preparation of bacterial cell-free extracts is described. The method involves the passage of a paste of bacteria and powdered glass between concentric cones of heavy glass. The inner cone is revolved by a motor while the outer cone is held firmly in place. After grinding, the resulting paste is extracted with buffer solution or water and the extract freed of glass, intact cells, and cell fragments by centrifugation.

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# BACTERIOLOGICAL STUDIES IN ENDOCARDITIS<sup>1</sup>

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Febrile disease associated with inflammatory lesions in the lining of the heart is properly designated as endocarditis. Most of our precise information concerning this group of disorders has been obtained by anatomical studies beginning at the necropsy. Two etiological types of endocarditis have thus been recognized, the bacterial and the nonbacterial, but even this distinction has remained somewhat indefinite. In the group of diseases included under the title of bacterial endocarditis various microbes have been recognized, but there has been no very great incentive to study the bacteria in detail because, until very recently, the outlook for life of the patient has been regarded as practically hopeless in any event, and the identification of the bacterial agent and detailed knowledge of its nature and peculiarities were relegated to the field of academic interests. Thus Perry (1936) in his monograph tabulated 1,000 cases of well-authenticated bacterial endocarditis, reported in the literature from 1899 to 1933. The bacteria concerned included 16 or more varieties. Even so the streptococcal infections were all included under four captions, (1) *Streptococcus*, (2) *Streptococcus haemolyticus*, (3) *Streptococcus nonhaemolyticus*, and (4) *Streptococcus viridans*. Evidently there has been very little interest in attempting to recognize different types within the viridans group. In this table there is no mention of *Streptococcus fecalis* (enterococcus), for example, although the occurrence of this organism as the infectious agent in bacterial endocarditis is clearly recognized by Lloyd-Jones in the chapter on experimental endocarditis included as an appendix in this same volume. More recently, however, the accumulation of information concerning anti-infectious agents has greatly increased the hope of successful therapy in bacterial infections of the blood stream and even of those in which there are bacterial foci in the endocardium. A more intimate study of the bacteria concerned and especially a study of their susceptibility and resistance to the various available anti-infectious agents may be expected to be of scientific interest and also of practical value as a guide in the attempt to overcome the infection.

Bacteriological study in a case of endocarditis is initiated by taking specimens of blood for culture. It is wise to take at least three such specimens on successive days and preferably at different hours, without awaiting the result of the first culture. Liquid and solid media should be inoculated with measured amounts

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of the citrated blood. If the patient has already received antibacterial treatment, the proper inactivating agent should be used in the cultures. When bacterial colonies have appeared, it is well to pick several of these for comparative

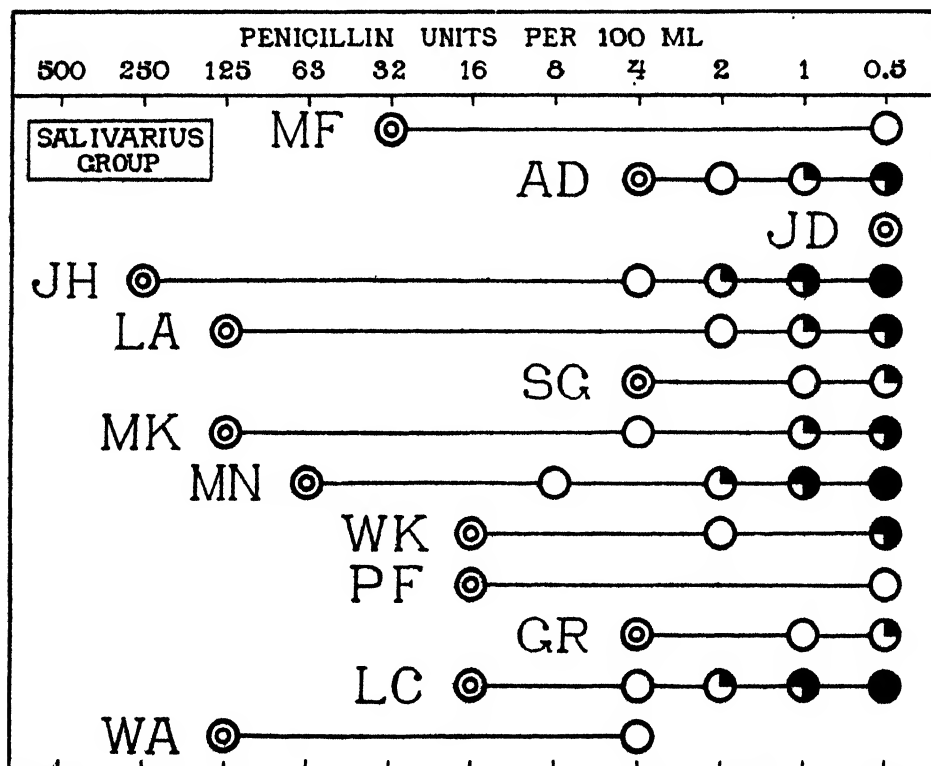


FIG. 1. THE EFFECT OF PENICILLIN IN THE TEST TUBE UPON CULTURES OF THE 13 BACTERIAL STRAINS OF THE SALIVARIUS GROUP

The double circle indicates "bactericidal effect." For example, the M.F. strain of *Streptococcus salivarius* inoculated into broth containing penicillin, 32 units per 100 ml of medium, failed to cause any perceptible clouding of the medium after 18 hours at 37 C, and the transfer of 0.1 ml from this tube to a fresh culture tube failed to produce any visible growth in 24 hours. In a primary culture containing 0.5 units penicillin per 100 ml of medium, the strain M.F. failed to produce a perceptible cloud in 18 hours, but the transfer from this tube to new medium did give rise to positive growth. This "bacteriostatic effect" is everywhere indicated by the single hollow circle. In a similar way the quarter black indicates slight turbidity ("marked inhibition") in the primary culture, as for example in strain A.D. at a concentration of 2 units per ml. The three-quarters black indicates more pronounced growth in the primary culture but less luxuriant than the control, designated as "slight inhibition." The solid black circle indicates "no inhibition," or growth in the primary culture not distinguished from that in the control tube without penicillin. It will be noted that all these salivarius strains were inhibited in their growth to some degree by as little as 1 unit of penicillin in 100 ml of the culture medium.

study and identification. The subcultures may then be tested for their ability to ferment various carbohydrates, for their susceptibility to the lytic action of bacteriophages and to the bactericidal and bacteriostatic action of penicillin, and also for the bactericidal and bacteriostatic effects of various chemical agents.

During the past year we have examined the positive blood cultures of 36

patients with bacterial endocarditis. Of these there were 13 culture strains classified, according to carbohydrate fermentations (Bergey *et al.*, 1939), as *Streptococcus salivarius*, 7 as *Streptococcus equinus*, 6 as *Streptococcus faecalis* (enterococcus), 3 as *Streptococcus bovis*, 2 in the viridans group unspecified, 2 in the micrococcus group unspecified; 1 was recognized as *Staphylococcus aureus* and

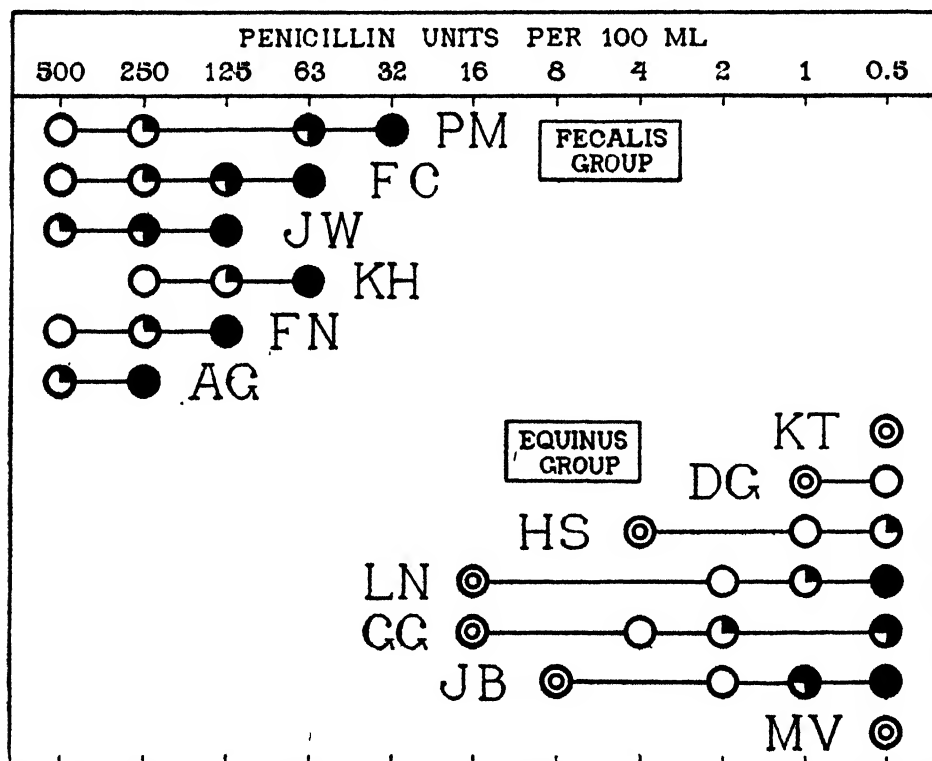


FIG. 2. THE EFFECT OF PENICILLIN UPON CULTURES OF THE 6 STRAINS IN THE FAECALIS (ENTEROCOCCUS) GROUP AND THE 7 STRAINS IN THE EQUINUS GROUP

Penicillin failed to show a "bactericidal" effect, even in a concentration of 500 units per 100 ml of medium, upon any of the faecalis strains and was not inhibitory at all in a concentration of 32 units per 100 ml. In contrast the equinus strains all showed the "bactericidal" effect in a penicillin concentration of 32 units per 100 ml, or less, and all revealed some inhibition in a concentration of 1 unit of penicillin per 100 ml of medium. Strains K. T. and M. V. were remarkably sensitive.

1 as an actinomyces, designated as *Actinomyces septicus*, n.sp.; and 1 gram-negative rod of undetermined species was found.

The organisms classed in the salivarius group were sensitive to penicillin in a concentration of one unit in 100 ml of culture medium. In one instance, strain J. D., the penicillin in a concentration of 0.5 units in 100 ml of culture medium exerted such a germicidal effect that, after exposure for 18 hours, the subculture failed to grow. In two other instances, M. F. and P. F., this amount of penicillin exerted a complete bacteriostatic effect. These three patients, J. D., M. F., and P. F., are still living, and in them the infection appears

to have been arrested. The equinus group contains only 7 strains and these seemed to be at least as susceptible to the penicillin.

In marked contrast was the behavior of the 6 strains in the faecalis group. None of these showed any evidence of growth inhibition by penicillin in a concentration of 32 units per 100 ml of culture medium, and none succumbed to a germicidal effect of penicillin even in a concentration of 500 units per 100

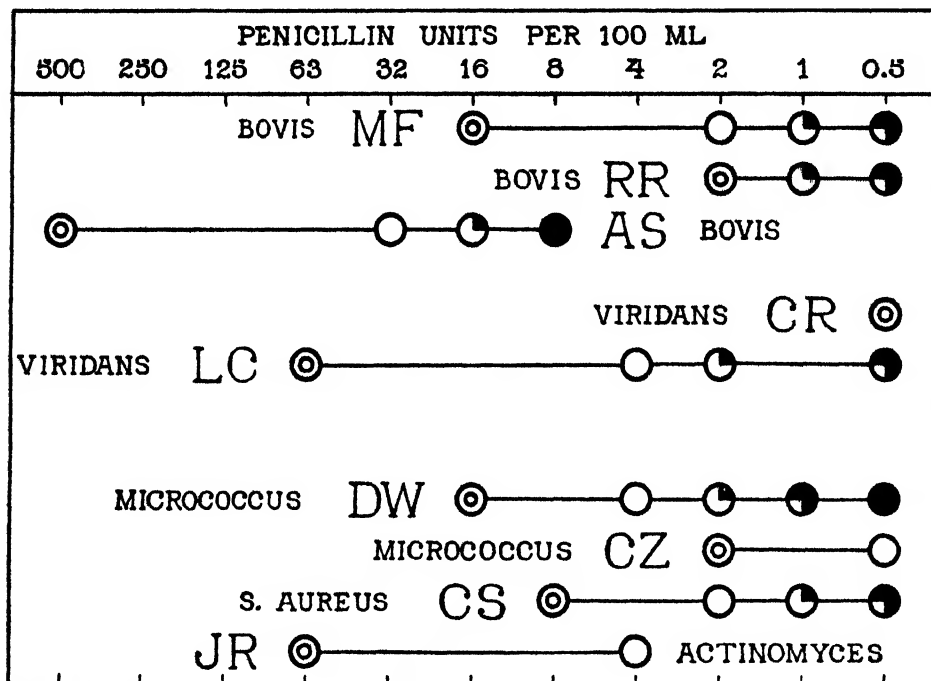


FIG. 3. THE EFFECT OF PENICILLIN ON 9 MISCELLANEOUS BACTERIAL STRAINS FROM THE BLOOD OF PATIENTS WITH ENDOCARDITIS

Note that none of these strains was so resistant as those in the faecalis group. The bovis strain A. S. was the most resistant of this assortment, showing no inhibition of growth in a concentration of 8 units of penicillin per 100 ml of medium. The viridans strain C. R. was most susceptible, showing the "bactericidal" effect in a concentration of 0.5 units of penicillin per 100 ml of medium. Note that the *Staphylococcus aureus*, C. S., was rather susceptible, and the actinomyces, J. R., rather resistant. The patient C. R. is dead; patients C. S. and J. R. survive.

ml. These streptococci in the faecalis group were found susceptible to lysis by bacteriophages available in our laboratory collection.

In the miscellaneous group, the organisms designated as *Streptococcus bovis* were variable in their behavior toward penicillin, and even the susceptible strain R. R. was still found in the aortic vegetations at necropsy. One of the unspecified viridans strains, C. R., was extremely susceptible to the action of penicillin even in a dilution of 0.5 unit per 100 ml of culture medium, but nevertheless this organism was also present in the aortic vegetations at necropsy. On the other hand, the *Staphylococcus aureus*, C. S., and the *Actinomyces septicus*,

J. R., were isolated from patients who have survived. Evidently susceptibility of the bacteria to the available therapeutic agents is a matter of importance but not the only factor in recovery of the patient.

The bacteriostatic effect of neoarsphenamine also proved to be variable, but there was no distinct difference between the faecalis group and the equinus group or the salivarius group as a whole. Partial inhibition of growth by 0.05 mg in 100 ml of culture medium was observed in two instances, strain L. C. in the

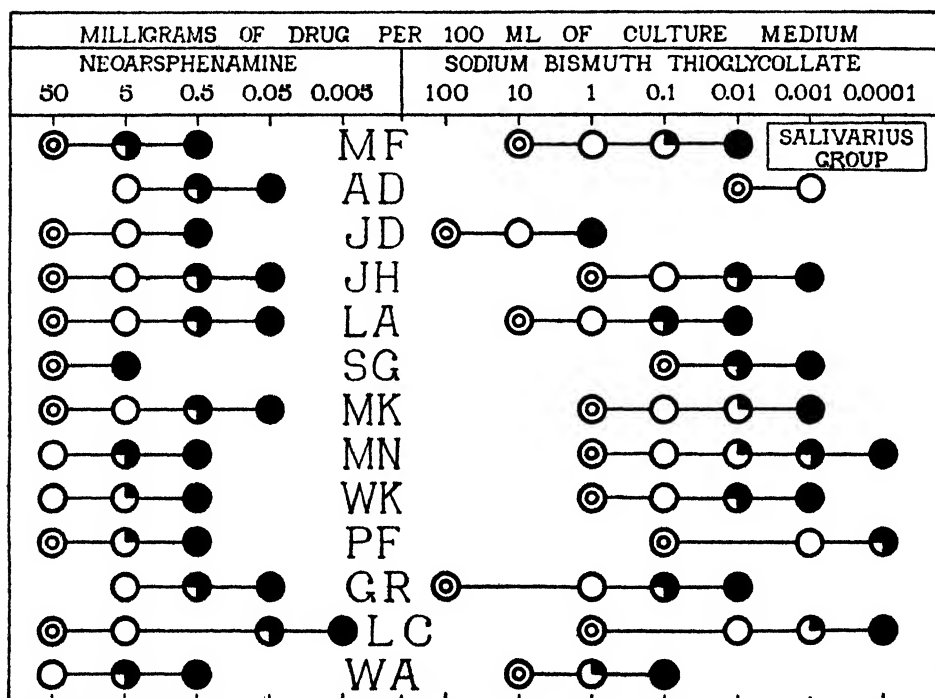


FIG. 4. THE EFFECT OF NEOARSPHENAMINE AND OF SODIUM BISMUTH THIOGLYCOLLATE (THIOBISMOL) ON CULTURES OF THE 13 SALIVARIUS STRAINS

The arsenical preparation exhibited slight inhibition in a concentration of 0.05 mg per 100 ml of medium against only one strain, L. C. The bismuth preparation was on the whole more effective and was almost unbelievably bacteriostatic in a dilution of 0.001 mg per 100 ml of medium against strains A. D. and P. F.

salivarius group and strain M. F. in the bovis group. Variation in the behavior toward bismuth in the form of thiobismol (sodium bismuth thioglycollate) was more marked. There was a germicidal effect in a dilution of 0.01 mg per 100 ml of medium for some strains (A. D., K. T., and R. R.) and for one strain (C. R.) in a concentration of 0.001 mg per 100 ml of medium. In other instances (strains J. D. and C. Z.) a concentration as great as 1 mg per 100 ml of medium was found to be without recognizable bacteriostatic influence. These differences are of considerable magnitude and should be taken into account in planning the antibacterial treatment of patients.

The possible development of drug fastness during the course of treatment is

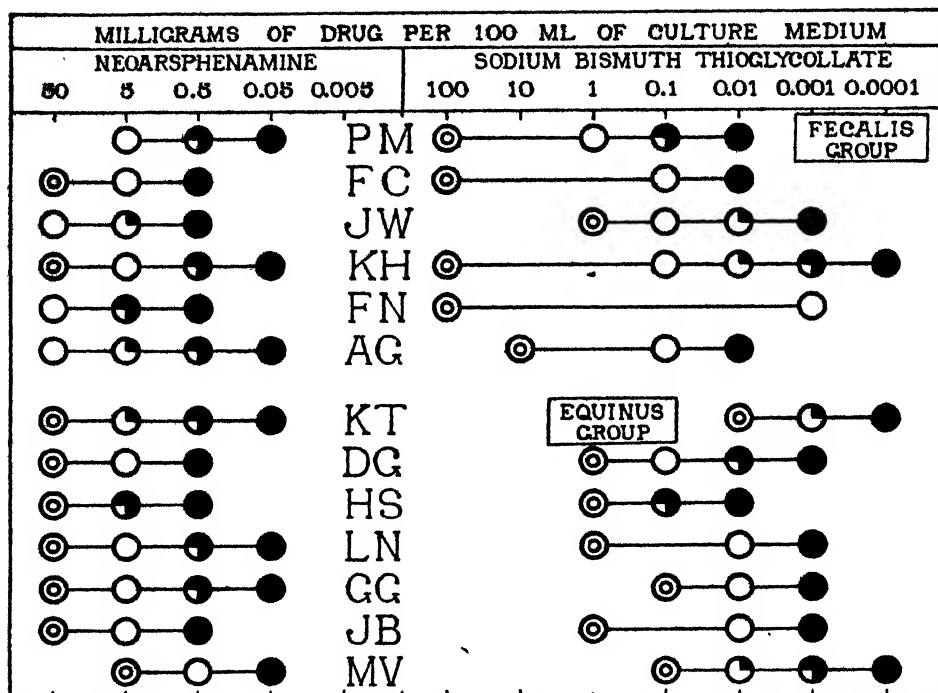


FIG. 5. THE EFFECT OF NEOARSPHENAMINE AND OF THIOMBISOL ON THE 6 STRAINS OF THE *Faecalis* GROUP AND THE 7 STRAINS OF THE *Equinus* GROUP

On the whole the *faecalis* group is evidently more resistant, but nevertheless one strain, F. N., was completely inhibited by 0.001 mg thiobismol in 100 ml of medium.

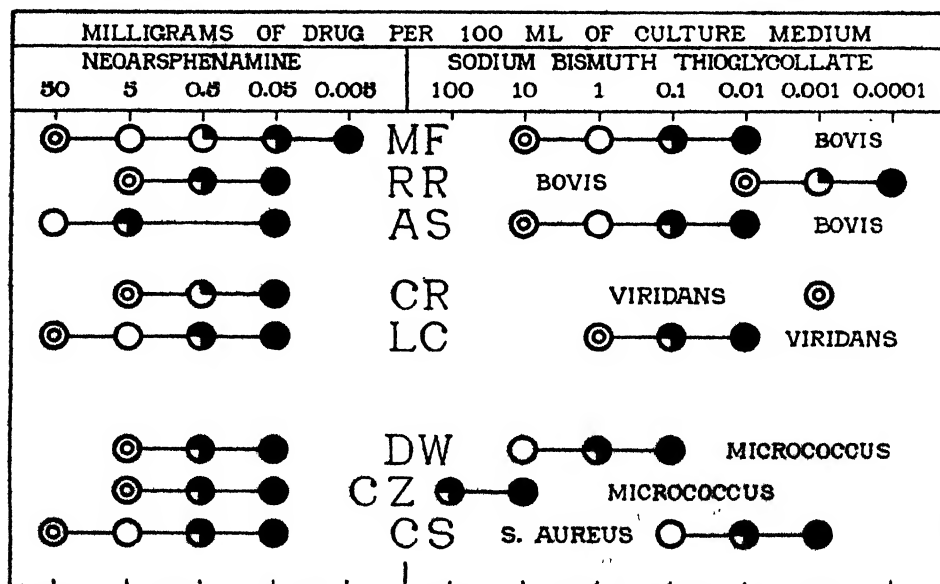


FIG. 6. THE EFFECT OF NEOARSPHENAMINE AND OF THIOMBISOL ON 8 MISCELLANEOUS STRAINS OF BACTERIA FROM THE BLOOD OF PATIENTS WITH ENDOCARDITIS

There are considerable differences among the strains, even among the three classed in the bovis group.

a matter not yet completely elucidated. When there has been favorable clinical response to treatment of an infection followed by relapse during continuation of the same therapeutic program, one suspects that the infecting microbe may have acquired a tolerance for the drug. Acquired microbic resistance of this sort has been demonstrated in a convincing manner for trypanosomes in rats and mice. In the study of acquired drug fastness by streptococci causing endocarditis there have been certain difficulties, particularly when the observations

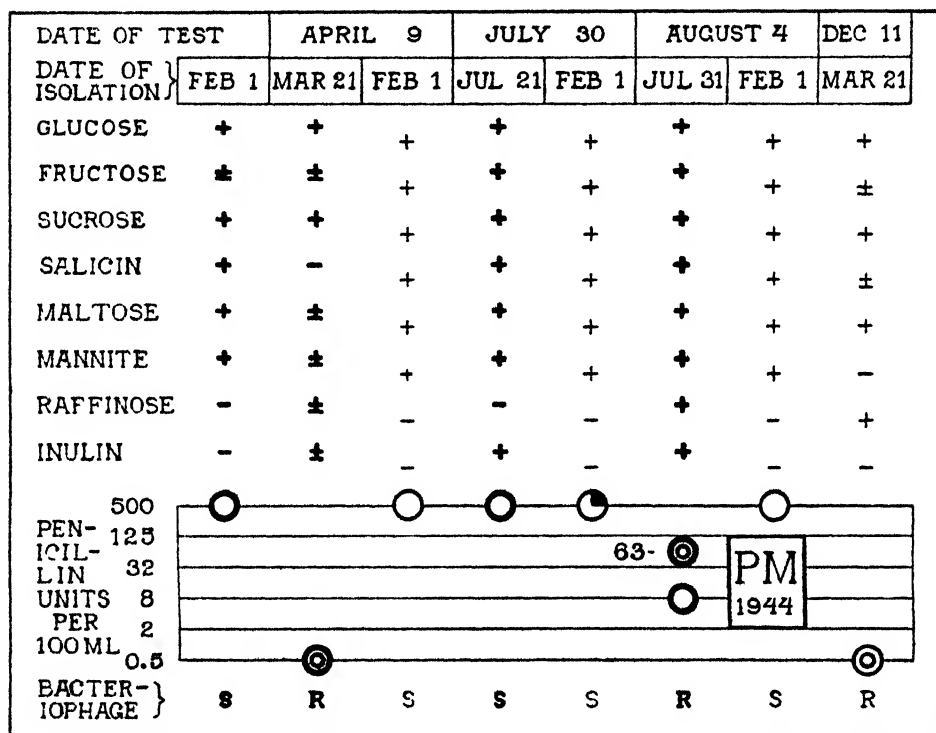


FIG. 7. LABORATORY TESTS OF BACTERIAL STRAINS ISOLATED FROM THE BLOOD OF P. M. ON FEBRUARY 1, MARCH 21, JULY 21, AND JULY 31

The results of the original first testing are shown with heavy lines, and the subsequent tests upon subcultures of the strains are shown by thinner lines. Note that the strains of February 1 and July 21 were resistant to penicillin and susceptible to bacteriophages, whereas the strain of March 21 was very susceptible to penicillin and wholly resistant to bacteriophages. The strain of July 31 seemed to be intermediate.

have been made only on the human disease. For example, in our patient P. M., now in the arrested stage of endocarditis, the bacteria obtained from the culture of February 1, 1944, required 500 units of penicillin per 100 ml of culture medium in order to inhibit bacterial growth, and these same bacteria were completely lysed by the enterococcus bacteriophage. A subsequent blood culture taken on March 21 yielded a streptococcus completely resistant to bacteriophage but susceptible to penicillin, so that 0.5 unit of this agent in 100 ml of culture medium was not only bacteriostatic but bactericidal for the organism. A few



months later a blood culture taken on July 21 yielded streptococci completely susceptible to the bacteriophage and resistant to penicillin. Somewhat similar variations in the character of the streptococci obtained in serial blood cultures have come to light in the study of other patients.

Observations of this sort challenge further investigation. Possibly the advent of the new bacterial type may be due to (1) gross technical error permitting the introduction of a foreign contaminant, (2) the temporary invasion of the patient's blood stream by the new streptococcus from a mucous surface, (3) the original presence of a double or multiple infection of the blood stream permitting the isolation of one bacterial type now and another later as the result of mere accident of subculture sampling or perhaps because of total or relative suppression of the earlier dominant type, (4) actual microbic variation in the descendants of a single bacterial cell, or (5) factors which still transcend our imagination. From the practical viewpoint in the selection of anti-infectious therapeutic agents, the careful study of the positive blood cultures obtained during the course of treatment would seem to offer helpful information.

#### SUMMARY

Detailed study of the bacteria isolated from the blood stream in endocarditis has come to be of practical significance since the infections in this category need no longer be summarily dismissed with a hopeless prognosis.

Use of ordinary morphological and cultural procedures, including the fermentation of carbohydrates, suffices to place these bacteria in various recognized groups.

The tests for resistance against biological and chemical anti-infectious agents permit the recognition of some remarkable differences in the culture strains and thus aid in determining the therapeutic program.

The enterococcus (*Streptococcus faecalis*) group is sharply distinguished by relative resistance to penicillin, but remarkable susceptibility to bacteriophages.

The question of acquired resistance to anti-infectious agents (drug fastness) requires further study.

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# ANTIBACTERIAL SUBSTANCES FROM PLANTS COLLECTED IN INDIANA

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The possibility that naturally occurring compounds showing pronounced antibiotic activity may be discovered in common plants offers an intriguing possibility for economical production of substances of interest and importance. During the summer of 1944 it was possible to collect and test substances from a number of plants in Indiana. It is the purpose of this report to give the details of this preliminary survey.

Osborn (1943) reported the results of examination of 2,300 samples from English plants and concluded that members of the Ranunculaceae offered most promise. Pederson and Fisher (1944) studied substances, in the juice of cabbage and other vegetables, which were active against gram-negative bacteria. Cavallito and Bailey (1944) demonstrated cysteine inactivation of active principles of various plant species. Lucas and Lewis (1944) reported preliminary results of a systematic survey of members of families of higher plants.

## EXPERIMENTAL

One or more samples of approximately 120 plant species were collected, mostly in Monroe County, Indiana, and transported to the laboratory in the usual ecological collection boxes. For the majority of specimens, the testing was done immediately or within 24 hours. When not used at once, the samples were refrigerated until time for testing. The exact list of species will be given later, but in the survey an attempt was made to include as great a variety of samples within the classification system as possible, yet some thought was given to the availability or ease of production of the species to be tested. Certain specimens were included because of previous interest, folklore or otherwise, in the medicinal value of the species. Deam (1940) was used as the authority for assignment of names of species, etc., except for a few cultivated varieties.

For testing, the juice of the specimen was expressed, without use of solvents, from the plant tissue by means of a Carver hydraulic press. Even in plants which were not especially succulent sufficient juice could be obtained by this method to supply the small amount needed for assay. The expressed juice was placed immediately, by means of a clean Wright pipette, into glass cylinders, as in the familiar Oxford cup (Abraham *et al.*, 1941) for assay of penicillin. The samples were placed in duplicate cups; one cup was on agar seeded with spores of *Bacillus subtilis* in approximately the concentration specified by Foster and Woodruff (1944). The second cup was on agar seeded with an 18-hour nutrient broth culture of *Escherichia coli*. The medium was the nutrient agar suggested by Schmidt and Moyer (1944) and the incubation temperature was

30 C. The results were usually observed at the end of 14 to 16 hours; a Quebec counter magnifying lens was used to improve accuracy in the measurement of the zones of inhibition or stimulation.

#### RESULTS

It is evident that many of the species showed some antibacterial substances for one or both of the test organisms. An additional group of plants, in contrast to these, produced a marked stimulation of the bacterial cultures as shown by a zone of more luxuriant growth in the region of the cup in comparison with that on the edge of the plate. Other samples showed no activity. For convenience, the exact values have been converted to the following symbols:

- S = zone of stimulation
- 0 = no activity
- +
- ++ = inhibitory zone, 15.1 to 19.9 mm
- +++ = inhibitory zone, 20.0 to 24.9 mm
- ++++ = inhibitory zone, 25.0 to 30.0 mm
- \* = zone definite but inhibition incomplete

The symbol or symbols before the slant line (/) in the list indicate the results using *B. subtilis*; and the symbols after this mark refer to activity for *E. coli*. In the majority of cases the values for duplicate samples (specimens collected at different dates or several specimens of the same species collected on the same date at different stations) confirmed the value obtained for the first sample tested. These have not been indicated, but in the cases of disagreement between duplicate samples the range of values is shown. Similarly, when various portions of the plant were tested separately, the results have been combined unless different values were obtained.

The following list shows the plants collected and the results obtained: (1) ACANTHACEAE: *Dianthera americana* (dense-flowered water willow)—0/0. (2) ACERACEAE: *Acer negundo* (box elder)—0/0. (3) ALISMACEAE: *Sagittaria* sp. (arrowleaf)—0/S, 0. (4) ANACARDIACEAE: *Rhus copallina* (shining sumac)—0/S; *R. glabra* (smooth sumac)—0/0; *R. typhina* (staghorn sumac)—S/S. (5) ANONACEAE: *Asimina triloba* (papaw)—0/0. (6) APOCYNACEAE: *Vinca minor* (periwinkle)—0/0. (7) ARACEAE: *Arisaema triphyllum* (jack-in-the-pulpit) leaves, stem, green berries—S/S; *Symplocarpus foetidus* (skunk cabbage) leaves—0/0, green fruit—0/+. (8) ASCLEPIADACEAE: *Ampelamus albidus* (bluevine)—S/S; *Asclepias verticillata* (horsetail milkweed)—0/0. (9) BALSAMINACEAE: *Impatiens pallida* (touch-me-not)—S/S. (10) BIGNONIACEAE: *Campsis radicans* (trumpet creeper)—0/0. (11) CAPRIFOLIACEAE: *Sambucus canadensis* (elderberry)—0/0. (12) CELASTRACEAE: *Celastrus scandens* (bittersweet)—0/S, 0; *Evonymus atropurpureus* (wahoo)—0/0. (13) COMPOSITAE: *Ambrosia elatior* (common ragweed)—++++, ++, 0/++++, 0/S; *A. trifida* (giant ragweed)—0/0; *Arctium minus* (burdock) leaves, flowers, stem—++++, ++/S, ++; *Cachia suaveolens* (Indian plantain)—S/+, +++; *Cichorium intybus* (chicory)—+/+, 0; *Cirsium arvense* (Canada thistle)—0/0; *C. discolor*

(field thistle)—S/0; *Eupatorium perfoliatum* (boneset)—0/0; *E. purpureum* (joey weed)—+\*/+\*; *Galinsoga ciliata* (quickweed)—0/0; *Helianthus mollis* (ashy sunflower)—++/0; *H. tuberosus* (Jerusalem artichoke)—0/0; *Heliopsis helianthoides* (sunflower heliopsis) all but root—0/0; *Ratibida pinnata* (cone-flower)—S/0; *Rudbeckia subtomentosa* (sweet cone-flower)—0/0; *R. triloba* (brown-eyed Susan)—S/0; *Silphium integrifolium* (entire-leaf rosinweed)—S/S; *S. laciniatum* (compass plant)—S/S; *S. perfoliatum* (cup rosinweed)—0/0; *S. terebinthinaceum* (dock rosinweed)—0, S/S; *Tanacetum vulgare* (common tansy)—0/0; *Taraxacum palustre* (dandelion)—0/0; *Xanthium pennsylvanicum* (cocklebur)—+/0. (14) CONVULVULACEAE: *Cuscuta* sp. (dodder)—+/0. (15) ERICACEAE: *Gaultheria procumbens* (wintergreen)—0/0; *Gaylussacia baccata* (black huckleberry)—S/S; *Vaccinium vacillans* (dry-land blueberry)—0/0. (16) EUPHORBIACEAE: *Acalypha virginica* (three-seeded mercury)—0/0, S; *Euphorbia maculata* (nodding spurge)—S/S; *E. marginata* (snow-on-the-mountain)—0/0; *E. supina* (spurge)—+\*/++++. (17) GRAMINEAE: *Digitaria sanguinalis* (crab grass)—S/S; *Eleusine indica* (goose grass)—S/S. (18) IRIDACEAE: *Iris* sp. (Iris)—0/0. (19) JUGLANDACEAE: *Carya* sp. (hickory)—0/0; *C. laciniosa* (big-leaf shagbark hickory)—S/0; *Juglans nigra* (black walnut)—+/0. (20) LABIATAE: *Lycopus americanus* (American bugleweed)—0/0; *Monarda fistulosa* (bergamot)—S/S; *Nepeta cataria* (catnip)—S/0; *Physostegia virginiana* (Virginia false dragonhead)—0/0; *Prunella vulgaris* (selfheal)—0/0; *Teucrium canadense* (American germander)—0/0. (21) LAURACEAE: *Sassafras albidum* (sassafras)—0/0. (22) LEGUMINOSAE: *Apios americana* (potato bean)—0/0; *Cassia nictitans* (small-flower sensitive plant)—S/0; *Robinia pseudoacacia* (black locust)—0/S; *Strophostyles helvola* (trailing wild bean)—0/S; *Tephrosia virginiana* (Virginia goat's rue)—S/S. (23) LILIACEAE: *Allium cepa* (onion)—++, S/0; *A. sativum* (garlic)—++++/++++; *Asparagus officinalis* (garden asparagus)—0/0; *Convallaria majalis* (lily of the valley)—0/0; *Funkia subcordata* (day lily)—0/0; *Hemerocallis fulva* (day lily)—0/0; *Smilacina stellata* (starry false Solomon's-seal)—0/0. (24) MAGNOLIACEAE: *Liriodendron tulipifera* (tulip tree)—0/S. (25) MARTYNIACEAE: *Martynia louisianica* (unicorn plant)—0/+. (26) MENISPERMACEAE: *Menispermum canadense* (common moonseed)—S/S. (27) NYMPHAEACEAE: *Nymphaea odorata* (water lily)—0/0. (28) ONAGRACEAE: *Ludwigia alternifolia* (seedbox)—0/++\*; *Oenothera pycnocarpa* (evening primrose)—0/S; (29) OSMUNDACEAE: *Osmunda cinnamomea* (cinnamon fern)—0/0; *O. regalis* (royal fern)—S/S. (30) OXALIDACEAE: *Oxalis europaea* (lady's sorrel)—+/+++; *O. grandia* (great yellow wood sorrel)—++/+++. (31) PAPAYACEAE: *Carica papaya* (papaya)—S/S. (32) PHYTOLACCACEAE: *Phytolacca americana* (pokeberry) leaves, stems, flowers, greenberries—++, S/++, S. (33) PLANTAGINACEAE: *Plantago rugelii* (common plantain)—S/S. (34) POLYGONACEAE: *Polygonum arifolium* (halberd-leaf tearthumb)—0/0; *P. aviculare* (knot weed)—S/S. (35) POLYPODIACEAE: *Cystopteris fragilis* (brittle fern)—S/S; *Onoclea sensibilis* (sensitive fern)—S/S; *Pteridium latiusculum* (bracken)—0/0. (36) PRIMULACEAE: *Lysimachia nummularia* (moneywort)—0/0. (37) RANUNCULACEAE: *Berberis thunbergii* (Japanese barberry)—++, +/+++, +;

*Jeffersonia diphylla* (twinleaf) root—0/S; *Ranunculus repens* (buttercup)—S/S. (38) ROSACEAE: *Agrimonia parviflora* (small flower agrimony)—0/0; *Paeonia* sp. (peony)—S/S; *Prunus serotina* (Black cherry)—0/0; *Spiraea tomentosa* (hardhack)—0/+++. (39) RUBIACEAE: *Cephalanthus occidentalis* (hairy buttonbush)—+++/++++. (40) SALICACEAE: *Salix babylonica* (weeping willow)—0/0. (41) SAXIFRAGACEAE: *Heuchera americana* (alumroot)—0/0; *Hydrangea arborescens* (smooth hydrangea)—+\*/0. (42) SCROPHULARIACEAE: *Mimulus alatus* (monkey flower)—0/0. (43) SIMARUBIACEAE: *Ailanthus altissima* (tree of heaven) male and female plants—S/S. (44) SOLANACEAE: *Datura stramonium* (Jimson weed) leaves and stem—S/S; *Physalis subglabrata* (smooth ground cherry)—++/++, S; *Solanum carolinense* (horse nettle)—++/0; *S. dulcamara* (bittersweet nightshade)—0,S/0,S; *S. nigrum* (common nightshade)—S,0/S,0. (45) TILIACEAE: *Tilia heterophylla* (white basswood)—0/0. (46) TYPHACEAE: *Typha latifolia* (common cattail)—0/0. (47) ULMACEAE: *Ulmus americana* (American elm)—0/0. (48) UMBELLIFERAE: *Cicuta maculata* (water hemlock)—0/0; *Daucus carota* (Queen Anne's lace; wild carrot)—0/S. (49) URTICACEAE: *Boehmeria cylindrica* (false nettle)—0/0. (50) VIOLACEAE: *Viola* sp. (Violet) leaves and petioles—0/0. (51) VITACEAE: *Parthenocissus quinquefolia* (Virginia creeper)—0/0.

#### DISCUSSION

From the foregoing list it can be seen that, although the juice of several species showed antibacterial activity, no specimen was encountered which gave exceptionally high values. It must be remembered, however, that with this method of assay it would be necessary for the substance to be of such a nature that it would diffuse easily through the agar. It is possible that samples which show some activity by this method would be found to have a greater potency when tested by another method. Similarly, the method of preparation of the sample juice did not include extraction of substances by organic solvents, and it seems probable that compounds might be present which would appear only under such conditions. In a few samples, particularly with *Cephalanthus occidentalis*, two zones of inhibitory activity against *B. subtilis* were pronounced; this would indicate the probability of more than one compound. No information is available yet on the separation of these.

It is believed that the inhibitory activity of some samples may be explained easily by the presence of well-known substances which are known to be toxic (such as oxalic acid, etc.) and may not be due to some new substance. This might apply to *Allium*, *Oxalis*, etc. The two species of *Ambrosia* which were tested gave different results, and this was true of several repeat samples; *A. trifida* consistently failed to show any activity against either test organism whereas *A. elatior* gave strong inhibition in almost every sample tested.

The high percentage of samples showing stimulation of the test organisms is perhaps worthy of mention. When it occurred, the phenomenon was usually so striking as to argue against the probability of a simple nutrient being the only stimulatory factor. The basal medium used for seeding is fairly adequate in

nutrients; thus the plant juices may have supplied growth factors or similar compounds. It would seem that an investigation of this phenomenon might yield fruitful results.

#### SUMMARY

The results are reported of a preliminary survey of antibacterial substances in a series of 120 or more plant samples collected in Indiana during the summer of 1944. The juice of the plants, or particular portions of them, obtained by a Carver hydraulic press, was tested for inhibitory activity against *Bacillus subtilis* and *Escherichia coli* with the Oxford cup technique.

Representatives (1 to 50 specimens) of the following families were included: Acanthaceae, Aceraceae, Alismaceae, Anacardiaceae, Anonaceae, Apocynaceae, Araceae, Asclepiadaceae, Balsaminaceae, Bignoniaceae, Caprifoliaceae, Celastraceae, Compositae, Convolvulaceae, Ericaceae, Euphorbiaceae, Gramineae, Iridaceae, Juglandaceae, Labiatae, Lauraceae, Leguminosae, Liliaceae, Magnoliaceae, Martyniaceae, Menispermaceae, Nymphaeaceae, Onagraceae, Osmundaceae, Oxalidaceae, Papayaceae, Phytolaccaceae, Plantaginaceae, Polygonaceae, Polypodiaceae, Primulaceae, Ranunculaceae, Rosaceae, Rubiaceae, Salicaceae, Saxifragaceae, Scrophulariaceae, Simarubiaceae, Solanaceae, Tiliaceae, Typhaceae, Ulmaceae, Umbelliferae, Urticaceae, Violaceae, Vitaceae.

Although about one tenth of the specimens showed some degree of inhibitory activity against one or both test organisms, no sample was encountered which gave exceptionally high values. A marked stimulation of growth of the test organism was evident with many samples.

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# INVESTIGATION OF THE EXISTENCE AND NATURE OF RESERVE MATERIAL IN THE ENDOSPORE OF A STRAIN OF *BACILLUS MYCOIDES* BY AN INDIRECT METHOD

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Because of its high density and low permeability, the bacterial endospore is not suitable for direct cytological investigations, and little is known with certainty about its internal structure. In this and similar cases it is desirable to resort to indirect methods which, although not including tinctorial and microchemical tests, should, nonetheless, be considered as legitimate methods of cytology. As an example we may refer to the brilliant work of Mudd and Mudd (1927) on the wetting property of the cell surface of *Mycobacterium tuberculosis*. Their conclusions were later confirmed for the human strain by the direct microchemical studies of the author (Knaysi, 1929).

Of considerable practical and scientific importance is whether the endospore contains reserve material and, if it does, what the nature of that material is. There is nothing in the literature to supply that much-needed information; one finds only statements that the reserve material of the sporangium gradually disappears during the maturation of the endospore, and that it may serve as nutritive material for the building of the spore (Meyer, 1899; Preisz, 1904); Lewis (1934) considered also the possibility that it may be utilized by the sporangium in its own metabolic processes.

The present work was inspired by the observation that endospores of strain C<sub>2</sub> of *Bacillus mycoides* washed several times in distilled water and resuspended in distilled water have no tendency to germinate. On the other hand, the addition of very small quantities of food, in the form of tryptone or meat infusion, to the suspension resulted in normal germination of most of the endospores within the expected time (Knaysi, 1945).

These early observations indicated clearly that endospores germinate only in the presence of utilizable food, and led us to the obvious corollary that an endospore containing reserve material both for energy and the synthesis of protoplasmic substances would tend to germinate when suspended in water, although the absence of an extraneous supply of food may make further growth impossible. The fact that endospores of the strain investigated had no tendency to germinate without an extraneous supply of utilizable food could mean either that they did not contain any reserve material or, if they did, that the reserve material was not suitable both as a source of energy and for the building of protoplasmic substance.

## METHODS

*Stock suspensions* of the endospores of the strain used in this investigation (strain C<sub>2</sub> of *Bacillus mycoides*) were prepared from 3-day-old cultures of the



organism at 33 C. The cultures were grown in large test tubes each containing 5 ml of the following medium: 100 ml of meat infusion diluted 4 times with distilled water + 0.25 g of tryptone + 1.5 g of bacto-agar, pH 7.0-7.2; or on that medium + 0.25 g of glucose. The endospores from a given culture were suspended in about 10 ml of sterile, distilled water, centrifuged down, resuspended in about the same volume of sterile, distilled water, recentrifuged down, etc. This process of washing was repeated 5 times over a period of 2 days. The final suspension was usually free of vegetative cells and could be kept in the laboratory for several weeks without significant change in the appearance and numbers of the endospores.

Numbers of the cellular elements per ml of the various media used were counted by the cover-glass method recently described (Knaysi, 1945).

In view of the fact that the organism investigated is able to utilize glucose as a source of energy and carbon, and  $\text{KNO}_3$  as a source of nitrogen, and that its washed endospores can germinate normally and grow into a culture when heavily inoculated into the medium (100 ml of distilled water + 0.2 g of glucose + 0.2 g of  $\text{KNO}_3$  + 0.23 g of an equimolar mixture of  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$ ), it was decided to use the components of this medium separately and in various combinations. We also used solutions in which lactose, a sugar not fermented by the organism, was substituted for glucose. The various solutions and concentrations used are recorded in the table.

Stock solutions of the various components were sterilized separately and mixed aseptically just before use. All test tubes used were thoroughly recleaned with dichromate cleaning solution and, after thorough rinsing with tap water then with distilled water, were plugged with fresh cotton and sterilized in the inverted position, care being taken that the plugs did not get soaked in water during sterilization. The chemicals used were of the grade "Baker's Analyzed, C.P." or equivalent brands, and the volume of each suspension was 10 ml. All suspensions were incubated at 33 C.

The pH was determined by the "spot plate" method, using the indicators of Clark and Lubs.

## RESULTS

The data recorded in the table show that the washed endospores of strains  $C_2$  of *Bacillus mycoides* have no tendency to germinate in distilled water or in the following aqueous solutions of the indicated strengths: an equimolar mixture of  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$ ; potassium phosphate +  $\text{KNO}_3$ ; or lactose + potassium phosphate +  $\text{KNO}_3$ . In addition to the lack of tendency to germinate in these media, one observes no change in the pH of the suspensions containing phosphate, and no significant change in the suspensions not containing a buffer. The pH of the latter suspensions was so unstable that we had difficulty in making accurate determinations.

*Typical germination* of the endospores into normal, rodlike vegetative cells and further growth of these cells were observed in the solutions containing glucose + potassium phosphate mixture, or glucose + potassium phosphate mixture +  $\text{KNO}_3$ , of the concentrations indicated in table 1. Indeed, in the first of the

TABLE 1

*Behavior of the washed endospores of Bacillus mycoides, strain C<sub>2</sub>, when suspended in the indicated media at 33 C*

COMPOSITION OF MEDIUM	AGE OF SUSPENSION		VEGETATIVE CELLS PER ML $\times 10^4$	NORMAL ENDO-SPORES PER ML $\times 10^4$	NONRE-FRACTIVE ENDO-SPORES PER ML $\times 10^4$	GERMINATING ENDO-SPORES PER ML $\times 10^4$	pH	REMARKS
	Hr	Min						
Distilled water	2	20	0.0	21.2	9.1	0.0	7.0	
	22	5	0.0	19.7	11.3	0.0	7.6	
	70	10	0.0	27.7	13.0	0.0	7.8	
Potassium phosphate mixture, 0.23 g % + KNO <sub>3</sub> , 0.2 g %	5	0	0.0	33.6	0.0	0.0	6.8	
	29	0		29.0	0.2	0.0	6.8	
Potassium phosphate mixture, 0.23 g %	5	15	0.0	24.5	1.2	0.0	6.9	
	29	30	0.0	32.4	0.7	0.0	7.0	
	81	30					7.0	
KNO <sub>3</sub> , 0.25 g %	5	30	0.0	54.5	2.4	0.0	8.2	Microscopic appearance unchanged
	24	10	0.0	67.6	2.9	0.0	7.6	
	0	0	0.0	41.0	1.2	0.0	7.6	
	5	0	0.0	39.6	0.7	0.0	7.2	
Lactose, 0.5 g %	3	5	0.0	24.5	11.0	0.0	7.8	
	5	50	0.0	24.5	12.7	0.0	7.8	
	23	50	0.0	25.7	17.5	0.0	7.8	
Lactose, 0.2 g % + phosphate, 0.23 g % + KNO <sub>3</sub> , 0.2 g %	18	25	0.0	27.6	11.8	0.0	6.8	Observations previous to this time revealed no change in microscopic picture
	93	40	0.0	28.3	10.3	0.0	6.8	
Glucose, 0.2 g %	0	0	0.0	60.5	4.6	0.0	8.0	Typical germination
	5	30	0.0	74.2	4.1	1.4	7.2	
	29	30	0.0	50.2	5.8	2.1	6.3	
	81	30	No change in microscopic picture				4.8	
Glucose, 0.2 g % + KNO <sub>3</sub> , 0.2 g %	5	15	0.2	41.6	4.1	2.9	7.2	Typical germination
	29	30	0.0	28.3	21.6	2.9	4.4	Nonrefractive spores are mostly shells
	81	30	No vegetative cells. Besides normal spores, many shrunken, refractive ones, apparently without exine or, occasionally, attached to an exine. Numerous shells and shell fragments.				4.0	Germination not typical, characteristic of low pH

TABLE 1—*Continued*

COMPOSITION OF MEDIUM	AGE OF SUSPENSION		VEGETATIVE CELLS PER ML $\times 10^4$	NORMAL ENDO- SPORES PER ML $\times 10^4$	NONREFRACTIVE ENDO- SPORES PER ML $\times 10^4$	GERMINATING ENDO- SPORES PER ML $\times 10^4$	pH	REMARKS
	Hr	Min						
Glucose, 0.2 g % + phosphate, 0.23 g %	5	0	0.0	16.8	0.0	7.2	6.9	Typical germination
	28	45	8.1	21.6	12.0	2.4	6.6	
	77	30	Nonmotile cocci, mostly large and normal, motile rods, and many sporangia.				6.4	
Glucose, 0.2 g % + phosphate, 0.23 g % + $\text{KNO}_3$ , 0.2 g %	0	0	0.0	50.4	10.2	0.0	6.9	Typical germination
	5	0	3.1	24.0	0.0	5.5	6.9	
	77	30	Typical culture with pellicle and many spores that did not germinate. No sporangia yet.				6.0	

Note. % = per 100 ml of water.

the two solutions numerous sporangia were observed in about 3 days; the suspension containing  $\text{KNO}_3$  in addition to glucose and phosphate contained no sporangia at the end of that period. This is additional evidence to that recently presented (Knaysi, 1945) indicating that endospores are formed by healthy cells facing starvation.

The behavior of the endospores in the solutions of glucose or of glucose +  $\text{KNO}_3$  (the concentrations are recorded in table 1) needs special consideration. In both solutions the endospores have a tendency to germinate; this is to be expected on the basis of the results reported in the preceding section. However, typical germination is observed only until the pH drops to a value somewhere between 5 and 6. Below this pH, the vegetative cells already formed are unable to survive, and typical germination is inhibited probably because of the destruction of potential cells in the early stages of germination. Finally, the cellular elements in such suspensions consist of a reduced number of unchanged spores with pairs and clumps of various sizes in which some still refractive, but apparently shrunken, endospores are found with nonrefractive spores, shells, and shell fragments; occasionally, a refractive body may be seen attached to a cracked exine. This phenomenon is particularly noticeable in the suspension containing glucose +  $\text{KNO}_3$  where the pH reaches a minimum of 4 or slightly below, and we have come to consider it as typical of a tendency to germinate at a pH too low for the survival of the organism. It also confirms our previous conclusion (Knaysi, 1945) that, in the presence of nutrients, endospores tend to germinate even in an environment where the germ cell cannot survive.

Before concluding this report of our observations, we wish to describe the behavior of certain endospores in suspensions where there is no tendency for

normal germination and growth. In those suspensions, a few endospores may very slowly become nonrefractive without a noticeable change in size. Although most of these spores may still be surrounded by an exine, their frequent presence in pairs and small groups, sometimes as tetrads, indicates a softening of the exine. Occasionally, the exine seems to have disappeared; these are virtually vegetative cells, and have a tendency to divide without significant growth, appearing as diplococci. This phenomenon needs further study, although we are now inclined to believe that the energy necessary for the slight change in the endospores may have been derived from a breakdown of the exine.

#### DISCUSSION AND CONCLUSIONS

The data presented in the preceding section and in the table show that endospores of the organism investigated are able to germinate typically into normal cells which are able to grow and form endospores in a medium containing only glucose and potassium phosphate, without a source of nitrogen. This indicates that the endospore contains a relatively considerable amount of a nitrogen-containing reserve material. The lack of tendency for normal germination and growth in solutions containing  $\text{KNO}_3$ , a suitable source of nitrogen, with or without phosphate, indicates that this reserve material is not suitable as a source of energy, and that the endospore contains no other reserve material suitable for that purpose. In view of the fact that the vegetative cells of the investigated organism are able to deposit fat as reserve material, the results of the present investigation mean that the reserve fat of the sporangium is not absorbed by the endospore, and that the endospore forms its own reserve material.

In comparing, at the end of 5 hours, the number of germinating spores in a glucose solution with the number of germinating spores in a solution of glucose + potassium phosphate, one is impressed by the accelerating effect of the phosphate; obviously, the phosphate does not act only as a buffer (see the review by Barron, 1943).

A question often asked in connection with the formation of endospores is: Is there a limiting concentration of nutrients below which endospore formation is not possible? In a previous report (Knaysi, 1945) we have shown that endospores can be formed on bacto-agar to which no nutrients have been added. The present investigation permits us to be more precise and state that, in the presence of a suitable source of energy, the nitrogenous reserve material present in the endospores of the strain investigated is sufficient for a completion of the cycle from spore to spore.

Of considerable interest is the minimum pH reached when endospores are suspended in unbuffered solutions of glucose (4.8) or, particularly, glucose +  $\text{KNO}_3$  (4.0 or slightly below). Suspensions of vegetative cells of the same strain in the latter solution reach a minimum pH of only 5.0 to 5.2. The difference must be sought not only in the low permeability of the exine, but also possibly in a greater concentration of nondiffusible substances in the endospore, as would be expected from a Donnan equilibrium. In any case, it is probable

that the internal pH of the vegetative cell in the solution of glucose +  $\text{KNO}_3$  at pH 5 is about the same as that of the endospore in a similar solution at pH 4.0. The value of that internal pH is of considerable importance and should correspond to the level of acidity at which the enzymes which take part in the fermentation of glucose are inhibited. The use of such solutions for testing sugar fermentation by the usually proteolytic organisms of the genus *Bacillus* recommends itself in preference to the complex media commonly used.

#### SUMMARY

The endospores of strain  $C_2$  of *Bacillus mycoides* washed five times in distilled water are able to germinate normally in a solution of glucose (0.2 g in 100 ml of water) without the addition of a source of nitrogen. When this glucose solution is buffered with potassium phosphate (0.2 g) at a pH of about 7, germination is followed by growth and sporulation. There is no tendency for germination in a solution of  $\text{KNO}_3$  (0.2 g + 100 ml of water) with or without potassium phosphate (0.23 g) unless glucose is added. It is concluded that endospores of the strain investigated contain relatively large amounts of a nitrogen-containing reserve material not suitable as a source of energy, and that they contain no other reserve material for that purpose.

It is shown that endospore suspensions in solutions of glucose or, particularly, glucose +  $\text{KNO}_3$  reach a minimum pH much below that reached by vegetative cells in similar solutions. New evidence is given to show that endospores are formed by a healthy cell facing starvation, and the question of a minimum concentration of nutrients for sporulation is discussed.

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## SOME EFFECTS OF PENICILLIN ON INTESTINAL BACTERIA

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From the very beginning of observations on the bacteriostatic activities of penicillin, it was noted that many of the gram-positive bacteria, including members of the genera *Staphylococcus*, *Streptococcus*, *Diplococcus*, *Clostridium*, and *Corynebacterium*, were extremely susceptible, whereas the gram-negative organisms, except for the meningococci and gonococci, were highly resistant to the bacteriostatic and antibacterial action of this agent. Resistance and susceptibility are, of course, relative terms; an organism may be resistant and entirely unaffected by concentrations of penicillin maintained in the blood stream, even when high doses are administered, but markedly affected by the concentrations which might be attained in other body fluids. As shown by Rammelkamp and Helm (1943), 2.5 Oxford units per ml of serum are rarely attained, and concentrations above 0.25 units per ml of serum are not maintained for any appreciable period of time, whereas distinctly higher concentrations may be maintained for longer periods in the bile. Concentrations of 50 to 300 units per ml are easily obtained in urine, provided there does not exist an active infection with penicillin-destroying organisms, and it is common practice to irrigate infected areas with penicillin solutions containing 250 or more Oxford units per ml. Many of the gram-negative bacteria, which are considered highly resistant when viewed from the standpoint of concentrations attainable and maintainable in the blood stream, might be amenable to the bacteriostatic action of the concentrations employed in irrigations and attained in urine.

That growth of some gram-negative bacilli is inhibited by penicillin has been noted from time to time. Thus Abraham *et al.* (1941) reported that concentrations 1,000 times greater than those effective against the highly susceptible streptococci, staphylococci, etc., were required for bacteriostasis against some members of the genera *Salmonella* and *Shigella*. Robinson (1943) noted that a penicillin solution which prevented growth of various gram-positive cocci when diluted 1:64,000 (viridans streptococcus) to 1:8,000,000, (hemolytic) was effective against *Salmonella enteritidis* in a dilution of 1:4,000 and that to bring about bacteriostasis in several other strains of *Salmonella* and *Shigella* a concentration of 1:1,000 was required. Hobby has recently (1944) submitted additional data on the bacteriostatic action of penicillin against a number of gram-negative bacilli. Typhoid and dysentery bacilli were relatively sensitive.

In table 1 are presented some observations on the the relative bacteriostatic

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effect of penicillin, employed for therapeutic use, against a number of stock (Army Medical School) and freshly isolated cultures of intestinal bacteria. Beef

TABLE 1  
*Growth of intestinal bacteria in nutrient broth containing penicillin*

ORGANISM Conc. (O.U. per ml) .....	VIGOR OF GROWTH*							
	100	50	20	10	5	2	1	None
<i>E. typhosa</i> .....	0	0	1	1	2	2	3	4
<i>S. paratyphi</i> .....	0	0	0	1	1	2	3	4
<i>S. schottmuelleri</i> .....	0	0	0	0	2	4	4	4
<i>S. cholerae-suis</i> .....	0	0	0	0	1	2	3	4
<i>S. abortivo-equi</i> .....	0	0	0	0	1	1	2	4
<i>S. enteritidis</i> .....	0	0	0	1	1	2	2	4
<i>S. pullorum</i> .....	0	0	0	0	2	3	4	4
<i>S. hersfeldii</i> .....	0	0	0	0	0	0	1	4
<i>S. typhi-murium</i> .....	0	0	0	0	1	3	4	4
<i>S. oranienburg</i> (case A) ..	0	0	0	2	3	4	4	4
<i>S. oranienburg</i> (case B) ..	0	0	1	3	4	4	4	4
<i>Proteus</i> (sp.) (urine).....	0	0	0	1	2	4	4	4
<i>Proteus</i> (sp.) (urine).....	0	0	1	2	3	4	4	4
<i>S. dysenteriae</i> .....	0	2	2	4	4	4	4	4
<i>S. ambigua</i> .....	0	0	0	1	1	2	4	4
<i>S. paradysenteriae</i> V .....	0	0	0	0	2	4	4	4
<i>S. paradysenteriae</i> W .....	0	0	2	4	4	4	4	4
<i>S. paradysenteriae</i> X .....	0	0	2	3	4	4	4	4
<i>S. paradysenteriae</i> Y .....	0	0	1	2	4	4	4	4
<i>S. paradysenteriae</i> Z .....	0	1	2	4	4	4	4	4
<i>S. newcastle</i> .....	0	3	4	4	4	4	4	4
<i>S. sonnei</i> .....	4	4	4	4	4	4	4	4
<i>E. coli</i> (stock) .....	0	4	4	4	4	4	4	4
<i>E. coli</i> (feces) .....	0	2	3	4	4	4	4	4
<i>E. coli</i> (feces) .....	0	1	2	4	4	4	4	4
<i>E. coli</i> (urine) .....	0	0	2	3	4	4	4	4
<i>E. communior</i> (water).....	2	4	4	4	4	4	4	4
<i>E. communior</i> (urine).....	2	4	4	4	4	4	4	4
<i>A. aerogenes</i> (feces) .....	1	3	4	4	4	4	4	4
<i>A. aerogenes</i> (sp. fl.) .....	4	4	4	4	4	4	4	4

\* Figures indicate relative vigor of growth.

extract broth free from glucose, to which was added penicillin in the concentrations designated, was distributed aseptically into test tubes and inoculated

with 0.05 ml of a 20-hour broth culture of the test organisms. Incubation was at 37 C and records of vigor of growth were made for various periods.

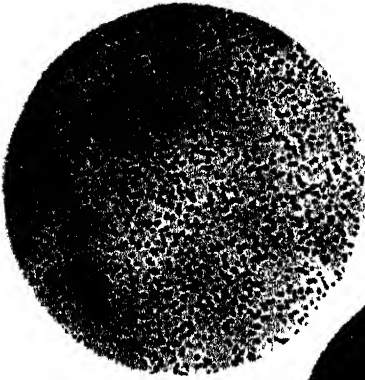
It will be noted that half of the *Salmonella* strains were distinctly inhibited in a relatively low concentration (2 units per ml); 6 of the 10 strains were completely prevented from growing and 2 others grew very poorly in 10 units of penicillin per ml. The culture of *Eberthella typhosa* was distinctly inhibited by two units per ml and markedly affected by 10 units of penicillin per ml, and the resistance of the two strains of *Proteus* was also comparable to what was observed for the varieties of *Salmonella*.

The strains of *Shigella* were much more resistant, generally requiring 10 to 20 units per ml to effect appreciable inhibition and 50 or more units per ml for complete bacteriostasis. *Shigella ambigua* was distinctly more susceptible and *Shigella sonnei* more resistant than the other strains of this genus.

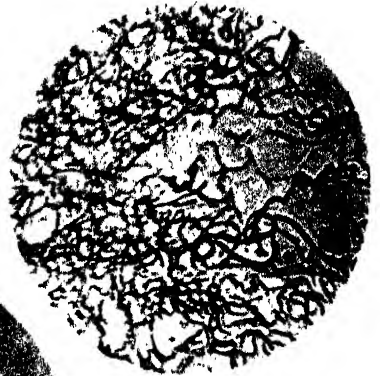
As a group, the strains of the genera *Escherichia* and *Aerobacter* were more resistant than those of the genus *Shigella*, the sucrose-negative *Escherichia* strains, however, being distinctly more susceptible than the sucrose-fermenting strains. With the exception of one strain of *Aerobacter*, growth of the coliform bacteria was markedly or completely inhibited by 100 units a concentration which can readily be attained in urine in the absence of penicillin-destroying bacteria. It would therefore appear that cystitis associated with some members of the genera *Escherichia*, *Aerobacter*, and *Proteus*, and with urinary carriers of typhoid and paratyphoid bacilli might be benefited by penicillin therapy.

Gardner (1940) noted striking changes in cultural and morphological characteristics among bacilli when growing in inhibitory concentrations of penicillin. Our observations with gram-negative intestinal bacteria disclosed that growth in liquid media, instead of being uniformly distributed throughout the liquid, was granular, restricted to the surface in some cultures and to the bottom in others. The butyrous consistency so characteristic of cultures of the intestinal group on nutrient agar was supplanted by a rubbery adherent growth as the penicillin concentrations increased. The morphological changes varied somewhat for the different organisms employed. Very bizarre involution forms were produced from long twisting filaments ten to several hundred times the normal cell in the lower inhibitory concentrations to such bizarre forms as cells resembling deformed integration signs, Pasteur flasks, swelled fusiform bacilli, large globular cells, and irregular masses resembling the pleomorphic forms described by Dienes and Smith (1944), when growth was present in higher concentrations. In some instances, examination of the sediment after centrifuging broth tubes containing concentrations of penicillin just above those permitting visible growth disclosed the presence of only large globular masses, which on transfer to broth (at intervals up to 10 days) yielded only typically small normal bacterial cells characteristic of the organisms in question. The involution forms described were readily visible from routine gram stain preparations, and staining with dilute carbol fuchsin frequently brought out structures which were not discernible in the gram stain. These involution forms were produced on agar as well as in liquid media.

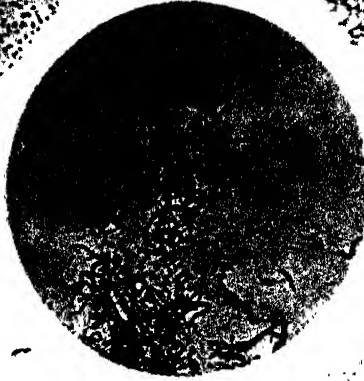




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2



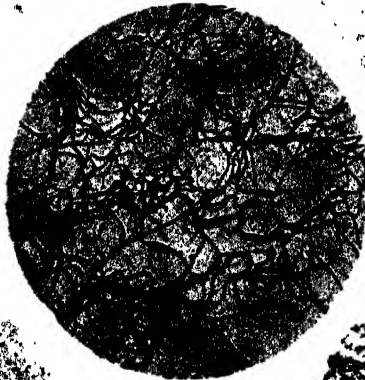
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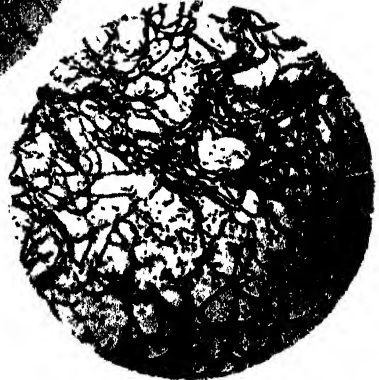
5



6



7



8

TABLE 2

*Photomicrographs showing effect of penicillin on morphology of gram-negative intestinal bacteria*

(Nutrient agar: 48 hr 37 C)

PHOTO NO.	GENUS	PENICILLIN (OXFORD UNITS PER ML)	MAGNIFICATION (DIAM )
1	<i>Proteus</i>	None	280
2	<i>Proteus</i>	10	330
3	<i>Proteus</i>	20	330
4*	<i>Proteus</i>	20	760
5*	<i>Proteus</i>	20	760
6	<i>Salmonella</i>	10	280
7	<i>Escherichia</i>	40	280
8	<i>Escherichia</i>	60	280

\* Selected fields from no. 3.

#### SUMMARY

The genera of the gram-negative bacilli of the intestinal tract seem to fall into the following order of descending susceptibility to penicillin: *Salmonella* and *Eberthella*, *Proteus*, *Shigella*, *Escherichia*, and *Aerobacter*.

Concentrations of penicillin readily attainable in urine (if actively penicillin-destroying bacteria are absent) are such as to warrant expectation of bacteriostatic effects on many species or varieties of the foregoing genera associated with urinary infections.

Changes in cultural characters and bizarre involution forms are produced by the gram-negative intestinal bacteria growing in liquid and solid media containing inhibitory, but not completely bacteriostatic, concentrations of penicillin.

The authors express their appreciation to Professor O. B. Williams of the University of Texas for the use of equipment and co-operation in the preparation of photomicrographs.

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# GROWTH OF *EBERTHELLA* TYPHOSA AND *AEROBACTER* AEROGENES IN ASSOCIATION IN TETRATHIONATE BROTH

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The observation by Muller (1923) of the value of sodium tetrathionate broth as a selective enriching medium for certain members of the enteric group of bacteria has been confirmed by a number of workers. Kaufmann (1930), for example, reported 30 per cent more positive isolations from specimens enriched in this medium than were obtained by direct plating, whereas Schaeffer (1935) obtained four times as many. Similar results by others have led to extensive use of the medium in public health laboratory work.

Ivanovics (1931), however, has reported observations which cast some doubt on the value of the medium. In one series of experiments he noted marked inhibition of *Eberthella typhosa* by each of 6 strains of *Aerobacter aerogenes* after incubation in mixed culture in sodium tetrathionate broth for 24 hours. With 3 of these strains no *Eberthella* colonies appeared on plates streaked from the mixture. In another series of experiments with 4 strains of *Aerobacter* he was unable to recover *Eberthella* in any instance from 24-hour mixed cultures of the two organisms. Only one of the *Aerobacter* strains which he used showed antagonistic action against *Eberthella* in plain broth, and in this one instance the effect was very slight. Since *Aerobacter* is of common occurrence in stool specimens, the serious practical import of the work of Ivanovics is obvious.

In connection with some other work we recently have repeated the experiments of Ivanovics, using a strain of *Aerobacter* which, unlike those strains tested by him, did show moderate antagonism in plain broth toward *Eberthella*. Both the *Aerobacter* and the *Eberthella* strains which were used have been maintained in stock culture for a number of years and have been found repeatedly, both before and during the present work, to be culturally and biochemically typical. The purity of each was established by picking from isolated colonies on eosin methylene blue agar plates.

The tetrathionate broth was prepared from a proteose peptone oxgall base. All other media were prepared from Difco dehydrated products.

The experimental method followed very closely that used by Ivanovics, differing only in that the observations were extended over a longer period of incubation and that a pure culture control of *Eberthella* in both plain and in tetrathionate broth was included. A loopful of a 24-hour nutrient broth culture of each species was planted in tubes of plain and of sodium tetrathionate medium. After agitation, a standardized loopful from each tube was spread on a plate of eosin methylene blue agar with a glass elbow rod. After some preliminary work it became possible to obtain regularly an initial ratio of *Eberthella* to *Aero-*

bacter of 1:1 to 1:2 as determined by counting from 400 to 600 colonies on plates which had been incubated for from 12 to 16 hours. Ordinarily no difficulty was encountered in differentiating the two species on the basis of colony appearance. In case there was doubt as to the identity of a colony type a transfer was made to double sugar medium for confirmation.

After incubation of the tubes for the intervals shown in the tabulation below, each culture was thoroughly shaken and a dilution made in sterile 0.85 per cent sodium chloride solution for streaking on eosin methylene blue agar plates. The proportion of culture to salt solution was varied when necessary so as to obtain about 300 colonies per plate. Growth in the tetrathionate medium was not so heavy as in the plain medium, and, consequently, 4 to 5 loopfuls of the tetrathionate culture were required to obtain the same number of colonies as would appear from the dilution of a single loopful of plain broth culture in an equal amount of salt solution. From these, plate counts were made to determine the effect of growth in association upon the ratio of *Eberthella* to *Aerobacter*. An equal amount of the pure culture of *Eberthella* was treated the same way in order to give a crude quantitative determination of the luxuriance of growth in pure culture. By comparison of the pure culture data with the mixed culture data it is possible to evaluate in a rough way the degree of antagonism exerted by *Aerobacter* in each medium provided one fact is taken into consideration, namely, that the total amount of growth of each organism in mixed culture is considerably less—roughly about one half—than that of either in pure culture, so that the total number of cells per unit volume of medium after incubation is approximately the same for both the pure culture and for the mixture. This fact was noted by Fulton (1937) for *Salmonella schottmuelleri* and *Escherichia coli* grown in association, and it has been repeatedly confirmed in connection with the present work. In the absence of antagonism two species of comparable growth rates grown in association should show approximately equal numbers of cells per unit volume of medium, and the total of the two should approximate the total growth of one grown in pure culture. If one species is antagonistic to the other, then the ratio of the one to the other at intervals of time may be interpreted as an indication of the degree of antagonism.

The results shown in table 1 are fully typical of those obtained.

The ratios given in column II support the statement that the strain of *Aerobacter* showed antagonism against the strain of *Eberthella* in plain broth. An initial ratio of 1:2 changed to a ratio of 1:300 over a 5-day period, and after 14 days no *Eberthella* colonies could be detected. These ratios, it will be recalled, are based upon a usual count of approximately 300 colonies. In column III appears the crude comparative quantitative count of the pure culture of *Eberthella*. At 24 hours, on the basis of the pure culture count, an *Eberthella* count from the mixture of between 150 and 200 would be predicted in the absence of antagonism. The ratio of 1:8 means an *Eberthella* count of about 35. Similarly at 48 hours and at 14 days the results speak for an inhibition of *Eberthella* by *Aerobacter*.

With tetrathionate broth the ratio values given in column IV taken alone

would indicate that the *Aerobacter* had little or no effect on the *Eberthella* since no decided change in ratio developed. Columns V and VI, however, indicate that antagonism in this medium did occur, since otherwise the counts in column VI should be roughly one half of the corresponding counts in column V. A comparison of the ratio values in columns II and IV, however, shows that the antagonism was much greater in plain broth than in tetrathionate medium. No significance can be attached to the tetrathionate results at the 14-day interval since by this time *Eberthella* had died out in this medium.

Although tetrathionate broth will not support so luxuriant a growth of either organism as will plain broth, it is initially more favorable to *Eberthella* than to *Aerobacter*, or else it exerts an initial selective inhibitory action on *Aerobacter*.

TABLE 1

*Growth of Eberthella in pure culture and in mixture with Aerobacter in plain and in sodium tetrathionate broth*

I TIME IN HOURS	II RATIO E/A IN PLAIN BROTH	III TOTAL EBER- THELLA. PURE CULTURE IN PLAIN BROTH	IV RATIO E/A IN TETRATHIONATE	V TOTAL EBER- THELLA. PURE CULTURE IN TETRATHIONATE	VI TOTAL EBER- THELLA. MIXED CULTURE IN TETRATHIONATE
0	1-2	—	1-1	—	—
24	1-8	400	1-3	500	50
48	1-14	600	1-2	700	120
72	1-100	—	1-3	900	100
96	1-150	—	1-2	650	150
120	1-300	—	1-3	350	110
*14 days	Pure A	150	Pure A	Sterile	Pure A

— not determined.

\* Inoculum at 14 days from plain broth was 4 to 5 times as great as at other intervals; from tetrathionate 4 to 5 times less than at other intervals because of heavy late multiplication of *Aerobacter*.

Whatever may be the explanation, the results obtained are exactly the reverse of the results reported by Ivanovics.

In view of the fact that the ratio of contaminants to significant bacterial type, after a short incubation period, is the factor of greatest importance in the practical use of tetrathionate medium, the results obtained are in favor of the use of the medium for enrichment purposes.

#### SUMMARY

A strain of *Aerobacter aerogenes* which was moderately antagonistic for *Eberthella typhosa* was found to show less antagonism in tetrathionate medium than in plain broth.

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## NOTE

### A BACTERIOPHAGELIKE PRINCIPLE FOR PSEUDOMONAS PYOCYANEA (PSEUDOMONAS AERUGINOSA)

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Received for publication April 9, 1945

A specimen of feces from a patient with acute colitis was forwarded to the laboratory for routine examination and culture. On MacConkey's medium several abnormal colonies of *Pseudomonas pyocyanea* were noticed.

The organism was isolated and subcultured on nutrient agar, and where growth was confluent the same abnormality previously noticed was present. In confluent areas of bacterial growth plaque formation similar to that found during bacteriophage activity in the *Salmonella* group was apparent.

Cultures in trypsin broth were prepared from the plaques and were rendered bacteria-free by Seitz filtration. A stock strain of *P. pyocyanea* which did not show plaque formation was treated with serial dilutions of the filtrate. Plaques developed in a maximum dilution of  $1/10^{-6}$ .

Secondary growth developed on the plaques. Subculture and treatment of these organisms with the phagelike filtrate indicated that some secondary growth organisms were lysable, whereas others were not lysable and grew normally.

These observations lead to the hypothesis that there is a bacteriophage for *P. pyocyanea* and that the phage can exist in susceptible and nonsusceptible strains.

Investigation of this problem is being continued and will be reported at a later date.





# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## EASTERN MISSOURI BRANCH

St. Louis, Missouri, February 20, 1945

ORAL MICROORGANISMS. I. THE TYPE OF ORAL MICROORGANISMS ISOLATED FROM MOUTH RINSINGS PLATED IN NUTRIENT AGAR. *Robert G. Sanders and George F. Reddish*, Bacteriology Department, Lambert Pharmacal Co., St. Louis, Mo.

Sterile water mouth rinses from 19 normal subjects were plated by serial dilution technique on bacto nutrient agar, and the morphology and cultural characteristics of more than 50 isolations were determined.

The most striking observation was the constant type of colonies observed from all individuals. Microscopic examination

showed that of the 50 isolations studied 90 per cent were micrococci. The other 10 per cent were made up of gram-negative rods, yeasts, and sarcina. Of the micrococci 60 per cent were gram-positive and 40 per cent were found to be gram-negative. It is evident that the micrococci represent a large and constant fraction of the bacterial flora of the normal mouth.

The carbohydrate fermentations of these oral micrococci were also determined. The possible relation of the production of acid from carbohydrates by these organisms to dental caries were discussed.



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